

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
REQUEST FOR FILING NATIONAL PHASE OF
PCT APPLICATION UNDER 35 U.S.C. 371 AND 37 CFR 1.494 OR 1.495

To: Hon. Commissioner of Patents
Washington, D.C. 20231

**

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)

Atty Dkt: 46 /220
M# /Client Ref.

From: Manelli Dension & Selter:

Date: May 16, 2001

This is a **REQUEST** for **FILING** a PCT/USA National Phase Application based on:

1. International Application <u>PCT/JP00/05210</u> ↑ country code	2. International Filing Date 03 August 2000 Day MONTH Year	3. Earliest Priority Date Claimed 17 September 1999 Day MONTH Year (use item 2 if no earlier priority)
---	--	---

4. Measured from the earliest priority date in item 3, this PCT/USA National Phase Application Request is being filed within:

(a) ☒ 20 months from above item 3 date (b) ☐ 30 months from above item 3 date,

(c) Therefore, the due date (unextendable) is 17 May 2001

5. Title of Invention: INHIBITOR AND ACTIVATOR FOR COUPLING FACTOR-6 AND USE THEREOF

6. Inventor(s) Tomohiro OSANAI and Koji MAGOTA

Applicant herewith submits the following under 35 U.S.C. 371 to effect filing:

7. ☒ Please immediately start national examination procedures (35 U.S.C. 371 (f)).

8. ☐ A copy of the International Application as filed (35 U.S.C. 371(c)(2)) is transmitted herewith (file if in English but, if in foreign language, file only if not transmitted to PTO by the International Bureau) including:

- a. ☐ Request;
- b. ☐ Abstract;
- c. pgs. Spec. and Claims;
- d. sheet(s) Drawing which are ☐ informal ☐ formal of size ☐ A4 ☐ 11"

9. ☒ A copy of the International Application has been transmitted by the International Bureau.

10. A translation of the International Application into English (35 U.S.C. 371(c)(2))

- a. ☒ is transmitted herewith including: (1) ☒ Request; (2) ☒ Abstract;
(3) 51 pgs. Spec. and Claims;
(4) 25 sheet(s) Drawing which are:
☒ informal ☐ formal of size ☒ A4 ☐ 11"
- b. ☐ is not required, as the application was filed in English.
- c. ☐ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
- d. ☒ Translation verification attached (not required now).

JC18 Rec'd PCT/PTO 1 6 MAY 2001

RE: USA National Filing Of PCT /JP00/05210

11. ☒ **PLEASE AMEND** the specification before its first line by inserting as a separate paragraph:
a. ☒ --This application is the national phase of international application PCT/JP00/05210 filed 3 August 2000 which designated the U.S.--
b. ☐ --This application also claims the benefit of U.S. Provisional Application No. 60/_____, filed _____--
12. ☐ Amendments to the claims of the International Application **under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., before 18th month from first priority date above in item 3, are transmitted herewith (file only if in English) including:**
13. ☒ PCT Article 19 claim amendments (if any) have been transmitted by the International Bureau
14. ☐ Translation of the amendments to the claims **under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., of claim amendments made before 18th month, is attached (required by 20th month from the date in item 3 if box 4(a) above is X'd, or 30th month if box 4(b) is X'd, or else amendments will be considered canceled).**
15. **A declaration of the inventor (35 U.S.C. 371(c)(4))**
a. ☐ is submitted herewith ☐ Original ☐ Facsimile/Copy
b. ☒ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
16. **An International Search Report (ISR):**
a. Was prepared by ☐ European Patent Office ☒ Japanese Patent Office ☐ Other
b. ☒ has been transmitted by the international Bureau to PTO.
c. ☒ copy herewith (3 pg(s).) ☐ plus Annex of family members (____ pg(s).).
17. **International Preliminary Examination Report (IPER):**
a. ☐ has been transmitted (if this letter is filed after 28 months from date in item 3) in English by the International Bureau with Annexes (if any) in original language.
b. ☐ copy herewith in English.
c.1 ☐ IPER Annex(es) in original language ("Annexes" are amendments made to claims/spec/drawings during Examination) including attached amended:
c.2 ☐ Specification/claim pages #____ claims #____
Dwg Sheets #____
d. ☐ Translation of Annex(es) to IPER **(required by 30th month due date, or else annexed amendments will be considered canceled).**
18. **Information Disclosure Statement including:**
a. ☒ Attached Form PTO-1449 listing documents
b. ☐ Attached copies of documents listed on Form PTO-1449
c. ☒ A concise explanation of relevance of ISR references is given in the ISR.
19. ☐ **Assignment** document and Cover Sheet for recording are attached. Please mail the recorded assignment document back to the person whose signature, name and address appear at the end of this letter.
20. ☐ Copy of Power to IA agent.
21. ☐ **Drawings** (complete only if 8d or 10a(4) not completed): ____ sheet(s) per set: ☐ 1 set informal;
☐ Formal of size ☐ A4 ☐ 11"
22. Small Entity Status ☒ is **Not** claimed ☐ is claimed (**pre-filing confirmation required**)
22(a) ____ (No.) Small Entity Statement(s) enclosed (since 9/8/00 Small Entity Statements(s) not essential to make claim)
23. **Priority** is hereby claimed under 35 U.S.C. 119/365 based on the priority claim and the certified copy, both filed in the International Application during the international stage based on the filing in (country) **JAPAN** of:
- | | Application No. | Filing Date | | Application No. | Filing Date |
|-----|-----------------|-------------------|-----|-----------------|-------------|
| (1) | JP 264687/1999 | 17 September 1999 | (2) | _____ | _____ |
| (3) | _____ | _____ | (4) | _____ | _____ |
| (5) | _____ | _____ | (6) | _____ | _____ |
- a. ☒ See Form PCT/IB/304 sent to US/DO with copy of priority documents. If copy has not been received, please proceed promptly to obtain same from the IB.
b. ☒ Copy of Form PCT/IB/304 attached.

RE: USA National Filing of PCT/JP00/05210

JC18 Rec'd PCT/PTO 1 6 MAY 2001

24. Attached: 1. Preliminary Amendment
2. Sequence Listing: [11] pages

25. Preliminary Amendment:

25.5 Per Item 17.c2, cancel original pages # _____, claims # _____, Drawing Sheets # _____

26. Calculation of the U.S. National Fee (35 U.S.C. 371 (c)(1)) and other fees is as follows:

Based on amended claim(s) per above item(s) ☐ 12, ☐ 14, ☐ 17, ☐ 25, ☐ 25.5 (hilitte)

Total Effective Claims	20	minus 20 =	x \$18/\$9	= \$0	966/967
Independent Claims	8	minus 3 =	x \$80/\$40	= \$400.00	964/965
If any proper (ignore improper) Multiple Dependent claim is present,			add \$270/\$135	+0	968/969

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)): →→ BASIC FEE REQUIRED, NOW →→→→

A. If country code letters in item 1 are not "US", "BR", "BB", "TT", "MX", "IL", "NZ", "IN" or "ZA"

See item 16 re:

- | | | |
|--|-------------------------|---------|
| 1. Search Report was <u>not</u> prepared by EPO or JPO ----- | add \$1000/\$500 | 960/961 |
| 2. Search Report was prepared by EPO or JPO ----- | add \$860/\$430 +860.00 | 970/971 |

SKIP B, C, D AND E UNLESS country code letters in item 1 are "US", "BR", "BB", "TT", "MX", "IL", "NZ", "IN" or "ZA"

- | | | | |
|--|-----------------|----|---------|
| (X) → <input type="checkbox"/> B. If USPTO did not issue <u>both</u> International Search Report (ISR) and (if box 4(b) above is X'd) the International Examination Report (IPER), ----- | add \$970/\$485 | +0 | 960/961 |
| (only) (one) → <input type="checkbox"/> C. If USPTO issued ISR but not IPER (or box 4(a) above is X'd), ----- | add \$710/\$355 | +0 | 958/959 |
| (these) (4) → <input type="checkbox"/> D. If USPTO issued IPER but IPER Sec. V boxes <u>not all</u> 3 YES, ----- | add \$690/\$345 | +0 | 956/957 |
| → <input type="checkbox"/> E. If international preliminary examination fee was paid to USPTO and Rules 492(a)(4) and 496(b) <u>satisfied</u> (IPER Sec. V <u>all</u> 3 boxes YES for <u>all</u> claims), ----- | add \$100/\$50 | +0 | 962/963 |

27. SUBTOTAL = \$1,260.00

28. If Assignment box 19 above is X'd, add Assignment Recording fee of ----\$40 +0 (581)

29. Attached is a check to cover the ----- TOTAL FEES \$1,260.00

**

Our Deposit Account No. 50-0687

Our Order No.	46	220
	C#	M#

CHARGE STATEMENT: The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-18 and 492 (missing or insufficient fee only) now or hereafter relative to this application and the resulting Official document under Rule 20, or credit any overpayment, to our Account/Order Nos. shown above for which purpose a duplicate copy of this sheet is attached.

This CHARGE STATEMENT does not authorize charge of the issue fee until/unless an issue fee transmittal form is filed

Manelli Denison & Selter
2000 M Street N.W., 7th Floor
Washington, DC 20036

By Atty:

Paul E. White, Jr.
Paul E. White, Jr.

Reg. No. 32,011

Sig:

Fax: 202-887-0336
Tel: 202-261-1050

Atty/Sec: /

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of

OSANAI, et al.

Group Art Unit: Not Assigned

Appln. No.: Not Assigned

Examiner: Not Assigned

Filed: May 16, 2001

Title: INHIBITOR AND ACTIVATOR FOR COUPLING
FACTOR-6 AND USE THEREOF

* * * * *

May 16, 2001

**PRELIMINARY AMENDMENT TO BE ENTERED PRIOR
TO CALCULATION OF FILING FEE**Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Please enter the following Preliminary Amendment of the subject
application prior to calculation of the filing fee of this application.

IN THE SPECIFICATION:

See the attached Appendix for the changes made to effect the below
paragraphs.

Page 1, paragraph starting at line 5

This invention relates to diagnostic methods and a diagnostic aid for
judging the onset and progress of diseases associated with a change in the
coupling factor (CF6) level in the blood, diseases in association with the excess or
shortage of PGI₂ or diseases associated with the accentuation or attenuation of
cPLA₂ function, which may be referred to as cPLA₂ hyperfunction or cPLA₂

09/831951

hypofunction and a pharmaceutical composition for the treatment of these diseases. The present invention further relates to a vector containing a DNA encoding CF6 or a polypeptide which is a part thereof, a transformant having been transformed by this vector, a method of efficiently producing CF6 and a partial polypeptide thereof, an antibody reacting specifically with CF6, a method for producing the antibody, and a method of assaying CF6.

Page 14, paragraph starting at line 8

The term "diseases associated with the excess of CF6" as used herein means diseases wherein the function of CF6 in the blood is accentuated to a level undesirable for a living body. Namely, these diseases are not always restricted to those wherein the blood CF6 level is higher than the level of normal persons. Namely, diseases associated with the shortage of PGI₂ and diseases associated with the attenuation of the cPLA₂ function or the cPLA₂ hypofunction fall within this category. Examples thereof include diseases associated with accentuated platelet agglutination, diseases associated with peripheral circulatory failure caused by inhibited vasodilatation, heart infarction, angina pectoris, heart failure, pulmonary hypertension, hypertension, cerebrovascular disorder, arteriosclerosis obliterans, arteriosclerosis, hyperlipemia, diabetes, bronchial disease, stomach ulcer, eclampsia of pregnancy, hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura.

Page 14-15, paragraph starting at line 26 of page 14

The term "diseases associated with the shortage of CF6" as used herein means

diseases wherein the function of CF6 in the blood is attenuated to a level undesirable for living body. Namely, these diseases are not always restricted to those wherein the blood CF6 level is lower than the level of normal persons. Namely, diseases associated with the excess of PGI₂ and diseases associated with the accentuation of the cPLA₂ function or cPLA₂ hyperfunction fall within this category. Examples thereof include brain infarction, acute pancreatitis, asthma, ARDS and rheumatoid arthritis.

IN THE CLAIMS:

Please amend claims 3, 6, 7, 9, 10, 12, 17 and 19 as follows (see the attached Appendix for the changes made to effect the below claims):

Claim 3. (Amended) A pharmaceutical composition for the prevention or the treatment as claimed in claim 1 wherein said disease associated with the excess of CF6 in the blood is heart infarction, angina pectoris, heart failure, pulmonary hypertension, hypertension, cerebrovascular disorder, arteriosclerosis obliterans, arteriosclerosis, hyperlipemia, diabetes, bronchial disease, stomach ulcer, eclampsia of pregnancy, hemolytic-uremic syndrome or thrombotic thrombocytopenic purpura.

Claim 6. (Amended) The pharmaceutical composition for the prevention or the treatment as claimed in claim 4 wherein said disease associated with the shortage of CF6 in the blood is an inflammatory disease such as brain

infarction, acute pancreatitis, asthma, ARDS or rheumatoid arthritis.

Claim 7. (Amended) A pharmaceutical composition for the prevention or the treatment of a disease associated with the shortage of PGI₂ and/or a disease associated with the attenuation of the Ca²⁺-dependent cytoplasmic PLA₂ (cPLA₂) hypofunction, which comprises a CF6 inhibitor as the active ingredient.

Claim 9. (Amended) A pharmaceutical composition for the prevention or the treatment as claimed in claim 7 wherein said disease associated with the shortage of PGI₂ and/or the disease associated with the the cPLA₂ hypofunction is heart infarction, angina pectoris, heart failure, pulmonary hypertension, hypertension, cerebrovascular disorder, arteriosclerosis obliterans, arteriosclerosis, hyperlipemia, diabetes, bronchial disease, stomach ulcer, eclampsia of pregnancy, hemolytic-uremic syndrome or thrombocytopenic purpura.

Claim 10. (Amended) A pharmaceutical composition for the prevention or the treatment of a disease associated with the excess of PGI₂ and/or a disease associated with the cPLA₂ hyperfunction, which comprises a CF6 activator or CF6 as the active ingredient.

Claim 12. (Amended) A pharmaceutical composition for the prevention or the treatment as claimed in claim 10 wherein said disease associated with the excess of PGI₂ and/or a disease associated with the cPLA₂ hyperfunction is an inflammatory disease such as brain infarction, acute pancreatitis, asthma, ARDS or rheumatoid arthritis.

Claim 17. (Amended) A diagnostic method of judging the susceptibility to a disease associated with an increase or decrease in the CF6 level in the blood, which involves the step of determining the presence/absence of a mutation in a gene sequence in the CF6 gene region in the genome of a subject.

Claim 19. (Amended) The diagnostic method as claimed in claim 17 wherein said disease with an increase or decrease in the CF6 level in the blood is a disease associated with the cPLA₂ hyperfunction or the cPLA₂ hypofunction.

REMARKS

This Preliminary Amendment revises the multiple dependent claims 3, 6, 9 and 12 to be single dependent claims in order to reduce the filing fee. The claims have been further amended as shown above to better recite patentable features of the present invention, the hypofunction and hyperfunction being supported in the present specification, respectively for example at page 5, line 4 and at page 18, line 10.

Additionally, the specification has been amended as shown above in order to correct clerical errors. The applicants submit that a person of ordinary skill in the art would understand the clerical nature of the errors when reading the present specification as a whole.

No new matter has been added.

Entry of this amendment and favorable consideration of this application are respectfully requested.

Respectfully submitted,

MANELLI DENISON & SELTER, PLLC

By Paul E. White, Jr.

Paul E. White, Jr.

Reg. No. 32,011

Tel. No.: (202) 261-1050

Fax No.: (202) 887-0336

2000 M Street, N.W.
Seventh Floor
Washington, D.C. 20036-3307
(202) 261-1000

APPENDIX**VERSION WITH MARKINGS TO SHOW CHANGES MADE****IN THE SPECIFICATION:**

Proposed Amendments To The Following Paragraphs Of The Specification
Showing Deletions And Insertions.

Page 1, paragraph starting at line 5

This invention relates to diagnostic methods and a diagnostic aid for judging the onset and progress of diseases associated with a change in the coupling factor (CF6) level in the blood, diseases in association with the excess or shortage of PGI₂ or diseases associated with the accentuation or attenuation of cPLA₂ function, which may be referred to as cPLA₂ hyperfunction or cPLA₂ hypofunction and a pharmaceutical composition for the treatment of these diseases. The present invention further relates to a vector containing a DNA encoding CF6 or a polypeptide which is a part thereof, a transformant having been transformed by this vector, a method of efficiently producing CF6 and a partial polypeptide thereof, an antibody reacting specifically with CF6, a method for producing the antibody, and a method of assaying CF6.

Page 14, paragraph starting at line 8

The term "diseases associated with the excess of CF6" as used herein means diseases wherein the function of CF6 in the blood is accentuated to a level undesirable for a living body. Namely, these diseases are not always restricted to those wherein the blood CF6 level is higher than the level of normal persons.

Namely, diseases associated with the shortage of PGI₂ and diseases associated with the attenuation of the cPLA₂ function or the cPLA₂ hypofunction fall within this category. Examples thereof include diseases associated with accentuated platelet agglutination, diseases associated with peripheral circulatory failure caused by inhibited vasodilatation, heart infarction, angina pectoris, heart failure, pulmonary hypertension, hypertension, cerebrovascular disorder, arteriosclerosis obliterans, arteriosclerosis, hyperlipemia, diabetes, bronchial disease, stomach ulcer, eclampsia of pregnancy, hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura.

Page 14-15, paragraph starting at line 26 of page 14

The term “diseases associated with the shortage of CF6” as used herein means diseases wherein the function of CF6 in the blood is attenuated to a level undesirable for living body. Namely, these diseases are not always restricted to those wherein the blood CF6 level is lower than the level of normal persons. Namely, diseases associated with the excess of PGI₂ and diseases associated with the accentuation of the cPLA₂ function or cPLA₂ hyperfunction fall within this category. Examples thereof include brain infarction, acute pancreatitis, asthma, ARDS and rheumatoid arthritis.

IN THE CLAIMS:

Proposed Amendments To Claims 3, 6, 7, 9, 10, 12, 17 and 19 Showing
Deletions And Insertions.

Claim 3. (Amended) A pharmaceutical composition for the prevention or the treatment as claimed in claim 1 [or 2] wherein said disease associated with the excess of CF6 in the blood is heart infarction, angina pectoris, heart failure, pulmonary hypertension, hypertension, cerebrovascular disorder, arteriosclerosis obliterans, arteriosclerosis, hyperlipemia, diabetes, bronchial disease, stomach ulcer, eclampsia of pregnancy, hemolytic-uremic syndrome or thrombotic thrombocytopenic purpura.

Claim 6. (Amended) The pharmaceutical composition for the prevention or the treatment as claimed in claim 4 [or 5] wherein said disease associated with the shortage of CF6 in the blood is an inflammatory disease such as brain infarction, acute pancreatitis, asthma, ARDS or rheumatoid arthritis.

Claim 7. (Amended) A pharmaceutical composition for the prevention or the treatment of a disease associated with the shortage of PGI₂ and/or a disease associated with the attenuation of the Ca²⁺-dependent cytoplasmic PLA₂ (cPLA₂) [function] hypofunction, which comprises a CF6 inhibitor as the active ingredient.

Claim 9. (Amended) A pharmaceutical composition for the prevention or the treatment as claimed in claim 7 [or 8] wherein said disease associated with the shortage of PGI₂ and/or the disease associated with the [attenuation of] the cPLA₂ [function] hypofunction is heart infarction, angina pectoris, heart failure, pulmonary hypertension, hypertension, cerebrovascular disorder, arteriosclerosis obliterans, arteriosclerosis, hyperlipemia, diabetes, bronchial disease, stomach

ulcer, eclampsia of pregnancy, hemolytic-uremic syndrome or thrombocytopenic purpura.

Claim 10. (Amended) A pharmaceutical composition for the prevention or the treatment of a disease associated with the excess of PGI₂ and/or a disease associated with the cPLA₂ hyperfunction, which comprises a CF6 activator or CF6 as the active ingredient.

Claim 12. (Amended) A pharmaceutical composition for the prevention or the treatment as claimed in claim 10 [or 11] wherein said disease associated with the [shortage] excess of PGI₂ and/or a disease associated with the cPLA₂ hyperfunction is an inflammatory disease such as brain infarction, acute pancreatitis, asthma, ARDS or rheumatoid arthritis.

Claim 17. (Amended) A diagnostic method of judging the susceptibility to a disease associated with an increase or decrease in the CF6 level in the blood, which involves the step of determining the presence/absence of a [variation] mutation in a gene sequence in the CF6 gene [domain] region in the genome of a [patient] subject.

Claim 19. (Amended) The diagnostic method as claimed in claim 17 wherein said disease with an increase or decrease in the CF6 level in the blood is a disease associated with the [accentuation or attenuation of] cPLA₂ [function] hyperfunction or the cPLA₂ hypofunction.

(19) 世界知的所有権機関
国際事務局



(43) 国際公開日
2001年3月29日 (29.03.2001)

PCT

(10) 国際公開番号
WO 01/21205 A1

- (51) 国際特許分類: A61K 45/00, 38/17, A61P 9/04, 9/12, 9/10, 3/06, 3/10, 11/00, 1/04, 7/00, 9/10, C12N 15/12, G01N 33/53
- (21) 国際出願番号: PCT/JP00/05210
- (22) 国際出願日: 2000年8月3日 (03.08.2000)
- (25) 国際出願の言語: 日本語
- (26) 国際公開の言語: 日本語
- (30) 優先権データ: 特願平11/264687 1999年9月17日 (17.09.1999) JP
- (71) 出願人 (米国を除く全ての指定国について): サントリー株式会社 (SUNTORY LIMITED) [JP/JP]; 〒530-8203 大阪府大阪市北区堂島浜2丁目1番40号 Osaka (JP).
- (72) 発明者: および
- (75) 発明者/出願人 (米国についてのみ): 長内智宏 (OS-ANAI, Tomohiro) [JP/JP]; 〒036-1322 青森県中津軽郡
- 岩木町大字駒越字村元31番1号 Aomori (JP). 孫田浩二 (MAGOTA, Koji) [JP/JP]; 〒569-1118 大阪府高槻市奥天神町2-14-38 Osaka (JP).
- (74) 代理人: 社本一夫, 外 (SHAMOTO, Ichio et al.); 〒100-0004 東京都千代田区大手町二丁目2番1号 新大手町ビル206区 ユアサハラ法律特許事務所 Tokyo (JP).
- (81) 指定国 (国内): AU, CA, CN, HU, JP, KR, US.
- (84) 指定国 (広域): ヨーロッパ特許 (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
- 添付公開書類:
— 国際調査報告書
- 2文字コード及び他の略語については、定期発行される各PCTガゼットの巻頭に掲載されている「コードと略語のガイダンスノート」を参照。

(54) Title: COUPLING FACTOR 6 INHIBITOR AND POTENTIATOR AND USE THEREOF

(54) 発明の名称: カップリングファクター6阻害剤および賦活剤ならびにその用途

(57) Abstract: Methods of determining the presence/absence of coupling factor 6 (CF6), which is a subunit of a proton-translocating ATPase occurring in mitochondria, in blood and measuring the concentration of the factor in blood by using a CF6 antibody, etc.; and techniques of treating various diseases by clarifying the relationships of the changes in the concentration of the factor in blood, production of prostacyclin in association with these changes, cPLA₂ activity, etc. with the diseases to thereby inhibit or potentiate the factor and diagnosing various diseases based on these findings. A process of efficiently producing CF6 or its fragment by using a vector containing a DNA encoding CF6 or its fragment.

[続葉有]

WO 01/21205 A1

704760 7307660



25/PRTS

09/831951
JC18 Rec'd PCT/PTO 1 6 MAY 2001

SPECIFICATION

INHIBITOR AND ACTIVATOR FOR COUPLING FACTOR-6 AND USE THEREOF

TECHNICAL FIELD

5 This invention relates to diagnostic methods and a
diagnostic aid for judging the onset and progress of
diseases associated with a change in the coupling factor
(CF6) level in the blood, diseases in association with the
excess or shortage of PGI_2 or diseases associated with the
10 accentuation or attenuation of cPLA_2 function, and a
pharmaceutical composition for the treatment of these
diseases. The present invention further relates to a
vector containing a DNA encoding CF6 or a polypeptide which
is a part thereof, a transformant having been transformed
15 by this vector, a method of efficiently producing CF6 and a
partial polypeptide thereof, an antibody reacting
specifically with CF6, a method for producing the antibody,
and a method of assaying CF6.

BACKGROUND ART

20 Prostanoids produced via the arachidonate cascade
(prostaglandin (PG), thromboxane (TG), leukotriene (LT),
etc.) are physiologically active substances which are
essential in maintaining the homeostasis *in vivo*.
Prostacyclin (PGI_2), which is a prostanoid is synthesized
25 in hemoendothelial cells under the action of PGI_2 synthase
on PGH_2 having been formed from arachidonic acid by
cyclooxygenase and prostaglandin H_2 (PGH_2) synthase.

It is known that the PGI_2 has the following

pharmacological effects:

(1) PGI₂ binds to PGI₂ receptor on membrane and activates adenyl cyclase to form cAMP, thereby exhibiting a platelet aggregation inhibitory effect and a vasodilating effect (Nakahata et al., Yakkyoku 50:1365-1373, 1999);

(2) PGI₂ plays an important role in thrombus formation caused by hemoendothelial disorder (Murata, T. et al., Nature 388:678-682, 1997); and

(3) PGI₂ is a major prostanoid inducing inflammatory edema (Murata, T. et al., Nature 388:678-682, 1997).

As described above, the biosynthesis pathway of PGI₂ and the enzymes participating in its biosynthesis (arachidonate cascade) have been identified and thus the physiological activities of PGI₂ have been clarified to a certain extent. However, the mechanism of regulating the PGI₂ production in diseases still remains unknown.

Regarding the regulation of the PGI₂ production, on the other hand, it has been suggested from the results of experiments with the use of spontaneously hypertensive rats (SHR), that an endogenous PGI₂ production inhibitor may be present in SHR (Osanai, T. et al., Jpn. Circ. J. 54, 507-514, 1990; Falardeau, P. et al., Prostaglandins, 29, 621-628, 1985). Although this endogenous PGI₂ production inhibitor has not yet been discussed sufficiently, the present inventors successfully isolated and purified from a SHR heart a PGI₂ production inhibitory factor secreted by smooth muscle cells originating in SHR mesenteric artery. Further, they determined the structure of this factor and

clarified that it is rat CF6 (hereinafter referred to as rCF6) which is one of the subunits of H⁺ ATP synthase (Referential Example 1).

Mammalian H⁺ ATP synthase is present in the
5 mitochondrial inner membrane and consists of at least 14 subunits. CF6, which is one of these subunits, consists of 76 amino acids. CF6 is synthesized as a peptide having 32 amino acids containing a mitochondrial transit sequence at the N-end (Higuchi, T. et al., Biochem. Biophys. Res.
10 Commun. 178, 793-799, 1991).

The present inventors have established a method comprising expressing a chimeric protein from which rCF6 can be cut off by using factor Xa with the use of *Escherichia coli* as a host, and cutting off rCF6 from the
15 chimeric protein followed by purification (Referential Example 2).

In addition, the present inventors have disclosed that the action point of the inhibition of the PGI₂ production by CF6 resides in the PLA₂ inhibition
20 (Referential Example 3), in particular, in the cPLA₂ inhibition (Referential Example 4) (Osanai, T. et al., J. Biol. Chem. 273, 31778-31778, 1998). However, the relationships between CF6, which is an intracellular peptide, and diseases have not been known so far. Moreover,
25 it still remains unknown as to how CF6 acts on hemoendothelium *in vivo*, namely, whether or not CF6 is present in the blood.

On the other hand, it has been clarified the

following facts with respect to the functions of cPLA₂.

(1) cPLA₂ is essentially required in the production of prostaglandin E₂, leukotriene B₄ and leukotriene C₄ which are inflammatory mediators in the arachidonate cascade; and

5 (2) a mouse lacking this enzyme shows a small infarcted area after tentatively ligating the middle cerebral artery and scarcely suffers from edema or nerve defect in the brain (Bonventre JV, T. et al. Nature 390:622-625, 1997).

10 Based on these facts, it is estimated that cPLA₂ inhibitors are usable as potent remedies for inflammation or remedies for brain infarction, etc. However, there has been found hitherto neither an efficacious remedy nor a method of screening a cPLA₂ inhibitor on the basis of the
15 effects of CF6.

As described above, the biosynthesis and physiological effects of PGI₂, the physiological effects of cPLA₂ in the arachidonate cascade and the relations among PGI₂, cPLA₂ and CF6 have been gradually clarified. However,
20 causes of diseases, in which PGI₂ located at the end of the arachidonate cascade and cPLA₂ located at the initial point thereof participate, have not been sufficiently clarified yet.

DISCLOSURE OF THE INVENTION

25 The present inventors have clarified that CF6 is present in rat blood and human blood and that CF6 is produced in hemoendothelial cells. They have also disclosed that the blood rCF6 level is elevated with an

increase in blood pressure in the case of hypertension with the shortage of PGI_2 ; that the blood pressure further increases by administering rCF6 in this state; and that an anti-rCF6 antibody exerts a hypotensive effect in the case of hypertension with a high rCF6 level. Moreover, they have found that the blood CF6 level is elevated in the case of human acute heart infarction, namely, the blood CF6 level changes in human diseases too, thereby having completed the present invention.

Under the above-described circumstances, the present invention relates to a pharmaceutical composition for the prevention or the treatment of diseases caused by changes in the blood CF6 level, diseases associated with the excess or shortage of PGI_2 and diseases associated with the accentuation or attenuation of cPLA_2 function, and diagnostic methods and a diagnostic aid for judging the onset and progress of diseases based on the control of the blood CF6 level.

Accordingly, the present invention provides a pharmaceutical composition for the prevention or the treatment of diseases associated with an increase or a decrease in the CF6 level, diseases associated with an increase or a decrease in the PGI_2 level and diseases associated with the accentuation or attenuation of the cPLA_2 function. The pharmaceutical composition comprises, as the active ingredient, CF6 activators (for example, CF6, CF6 secretion-promoting substances and CF6 agonists) or CF6 inhibitors (for example, CF6 secretion-inhibiting

substances and CF6 antagonists). The present invention also provides a method of measuring the blood CF6 level. The present invention still further provides a diagnostic method and a diagnostic aid for diseases associated with an
5 increase or a decrease in the CF6 level.

Moreover, the present invention provides CF6 secretion accelerating substances, CF6 secretion inhibitory substances, CF6 agonists and CF6 antagonists which are the active ingredients of the pharmaceutical composition for
10 the prevention or the treatment of the diseases as cited above. In addition, the present invention provides an anti-CF6 antibody which is usable as a reagent in the method of measuring the blood CF6 level or as a diagnostic aid in the diagnostic methods. The present invention
15 further provides a vector containing a DNA encoding CF6, a transformant having been transformed by this vector, and a method of producing a CF6-like peptide. According to the present invention, an antibody reacting specifically with CF6 or a partial polypeptide thereof and a method for
20 producing the same are provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram which illustrates how to construct a vector for expressing a chimeric protein from which rCF6 or human CF6 (hCF6) can be cut off with
25 enterokinase from a rat liver cDNA library or a human kidney cDNA library.

Fig. 2 shows the C18-HPLC elution profile of a solution wherein the rCF6-containing chimeric protein thus

expressed is treated with enterokinase.

Fig. 3 shows the C18-HPLC elution profile of partial purified rCF6.

Fig. 4 shows the C18-HPLC elution profile of a solution wherein the hCF6-containing chimeric protein thus expressed is treated with enterokinase.

Fig. 5 shows the C18-HPLC elution profile of partial purified rCF6.

Fig. 6 provides SDS-PAGE patterns of an antiserum prepared by administering to rabbits an immune antigen derived from rCF6, and of antibodies purified by the immunoaffinity chromatography using an antigen peptide as the affinity ligand.

Fig. 7 provides a chart which shows the effect of the anti-rCF6 antibody on the blood pressure of SHR (16 weeks of age).

Fig. 8 provides a graph which shows the effects of the anti-rCF6 antibody on rats (SHR and WKY, 16 weeks of age).

Fig. 9 provides a chart which shows the effects of the preadministration of the anti-rCF6 antibody on the decrease in blood pressure of rats (SHR, 16 weeks of age) caused by bradykinin.

Fig. 10 provides a chart which shows the effects of the preadministration of the anti-rCF6 antibody on the decrease in blood pressure of rats (SHR and WKY, 16 weeks of age) caused by bradykinin.

Fig. 11 provides a chart which shows the effects of

rCF6 on the blood pressure of rats (SHR and WKY, 16 weeks of age).

Fig. 12 provides a chart which shows the fractionation with Sephadex G-25 of a boiling water-extract of SHR heart.

Fig. 13 provides a chart obtained by analyzing the active fraction in the above Fig. 12 by HPLC.

Fig. 14 provides a chart obtained by analyzing the active fraction in the above Fig. 13 by HPLC.

Fig. 15 is a diagram which shows how to construct a vector for expressing a protein containing rCF6 having the recognition sequence of the factor Xa added to the N-terminus from a rat aorta cDNA library.

Fig. 16 provides a graph which shows the inhibition of the PGI₂ production by rCF6.

Fig. 17 provides a graph which shows the inhibition of production by rCF6 in the presence of bradykinin.

Fig. 18 provides a graph which shows the effect of rCF6 on the release of arachidonic acid from human umbilical venal endothelial cells (HUVEC).

Fig. 19 provides a graph which shows the effect of rCF6 on the effect of bradykinin of releasing arachidonic acid from HUVEC.

Fig. 20 provides a graph which shows the effects of various PLA₂ inhibitors and rCF6 on the release of arachidonic acid from HUVEC.

Fig. 21 provides a graph which shows the effects of various PLA₂ inhibitors and rCF6 on the effect of

bradykinin of releasing arachidonic acid from HUVEC.

Fig. 22 provides a graph which shows the effects of rCF6 on the blood pressure of rats (SHR and WKY, 16 weeks of age).

5 Fig. 23 provides a graph which shows a comparison of the blood hCF6 levels of normal subjects and patients with acute heart infarction.

Fig. 24 provides a graph which shows changes in the blood hCF6 level of patients with acute heart infarction.

10 Fig. 25 provides a graph which shows changes in the hCF6 level in a liquid culture of HUVEC.

MODE FOR CARRYING OUT THE INVENTION

(Production of CF6)

In the present invention the rat CF6 and the human
15 CF6, which are to be used as the active ingredients in the above-described pharmaceutical composition for the prevention or the treatment, as a reagent or a diagnostic aid in the method of measuring the blood CF6 level, or as a reagent or a diagnostic aid in the diagnostic methods for
20 diseases associated with changes in the blood CF6 level, can be efficiently produced by using enterokinase as a cutting enzyme compared with the conventional method (Referential Example 2). Enterokinase is an enzyme which recognizes a sequence Asp-Asp-Asp-Asp-Lys (SEQ ID NO:3) and
25 cuts the peptide bond at the C-end thereof. To obtain CF6, a chimeric protein carrying a protective peptide at the N-terminus of the enterokinase recognition sequence and CF6 or a partial peptide thereof at the C-terminus is obtained

by a conventional gene recombination technique and then CF6 is cut off therefrom with enterokinase. As the chimeric protein, use can be made, for example, of a peptide wherein CF6 is bonded via the recognition sequence of enterokinase to the C-terminus of a peptide consisting of 139 N-terminal amino acids of *E. coli* β -galactosidase (cysteine residues at the 76- and 122-positions have been substituted by serine: SEQ ID NO:4). Due to a large accumulation of the insoluble fraction of *E. coli*, this chimeric protein can be efficiently expressed and purified. After solubilizing the insoluble chimeric protein in a urea solution, CF6 can be cut off from the chimeric protein by treating with enterokinase. The thus cut off CF6 can be purified by subjecting to HPLC employing a C18 column. An expression vector can be constructed and a host can be transformed each by a conventional method well known by a person skilled in the art (Maniatis et al., "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory, 1989). (Partial polypeptide)

The term "partial polypeptide" as used herein means a polypeptide corresponding to a part of CF6 which has an effect of inhibiting the production of PGI_2 from human umbilical venal endothelial cells (HUVEC). Also, polypeptides formed by the modification of a polypeptide corresponding to a part of CF6 by the deletion, addition, substitution or combination thereof of one to several amino acids and having an effect of inhibiting the production of PGI_2 from HUVEC fall within this category.

(Production of CF6 antibody)

An antibody immunologically specific to CF6, which is to be used as the active ingredient in the above-described pharmaceutical composition for the prevention or the treatment or as a reagent or a diagnostic aid in the method of measuring the blood CF6 level or the diagnostic methods in the present invention, can be obtained by sensitizing an animal (preferably a non-human animal) with the use of CF6, its fragment or an analog thereof as an immunogen by a conventional experimental method. In case of preparing a monoclonal antibody, use can be made of, for example, the hybridoma method (Kohler, G. et al. Nature, 256:495-497, 1975) whereby an antibody produced by continuous cell line culture is presented. A single-stranded antibody against CF6 can be produced by applying a technique to be used in producing a single-stranded antibody (US Patent 4946778). Also, a human-type antibody can be expressed by using a transgenic mouse having a human antibody production system (Green LL. et al. Nat Genet, 7, 13-21, 1994). In this specification, an example of the production of a polyclonal antibody, wherein rabbits were immunized with an antigen obtained by synthesizing a peptide having a cysteine added to the N-terminus of an rCF6- or hCF6-origin fragment consisting of about 20 amino acids and bonding keyhole limpet hemocyanin thereto via the N-terminal cysteine, is presented (Example 2).

(Method of measuring CF6 level)

According to the present invention, CF6 in a

sample can be assayed by the RIA method or the ELISA method with the use of the antibody as described above (Radioimmunoassay: Kitagawa p.79-88; Enzyme Immunoassay:p.88-99, Shin-seikagaku Jikken Koza 12, Bunshi

5 Menekigaku III, Kogen, Kotai, Hotai, ed. by Nippon Seikagaku Gakkai, 1992). As an example thereof, an antagonistic inhibition system (RIA method) wherein quantification is carried out by inhibiting the reaction between a ¹²⁵I-labeled antigen with an antibody by an
10 antigen in a sample is presented in this specification (Example 2).

(Method of screening CF6 secretion accelerating substance or CF6 secretion inhibitory substance)

According to the present invention, it is also
15 possible to obtain a CF6 secretion accelerating substance or a CF6 secretion inhibitory substance by using the above-described method of measuring the CF6 level. More particularly speaking, a test substance is added to a liquid culture of cells capable of secreting CF6 (for
20 example, HUVEC) and the amount of CF6 secreted into the liquid culture is measured by an assay system with the use of the anti-CF6 antibody (for example, the RIA method as described above). Thus, a CF6 secretion accelerating substance or a CF6 secretion inhibitory substance can be
25 obtained (Example 7). It is still preferable to use cells causative of diseases as the cells capable of secreting CF6. (Method of screening CF6 agonist or CF6 antagonist)

According to the present invention, it is also

possible to obtain a method of detecting a CF6 agonist or a CF6 antagonist.

More particularly speaking, a test substance is added together with CF6 to a liquid culture of cells wherein the PGI₂ production is inhibited by CF6 (for example, HUVEC or smooth muscle cells originating in rat mesenteric artery). Then 6-keto-PGF1 α , which is the stable metabolite of PGI₂, secreted in the liquid culture is quantitated. Thus, a CF6 antagonist causing an increase in the 6-keto-PGF1 α amount or a CF6 agonist causing a decrease in the 6-keto-PGF1 α amount can be obtained. Alternatively, an antagonist or an agonist can be detected by adding a test substance together with CF6 to a liquid culture of HUVEC in the presence of labeled arachidonic acid and measuring arachidonic acid thus released into the medium. It is still preferable to use cells causative of diseases as the cells producing PGI₂. (Method of identifying CF6-binding receptor)

The present invention also provides a method of identifying a CF6-binding receptor. A CF6-binding receptor can be identified by standard receptor-binding methods commonly known in the art with the use of CF6. Although these methods include the ligand binding method and the crosslinking assay method, the present invention is not restricted thereto. In such a method, CF6 is fused with a peptide sequence appropriate for radioisotope (for example, ¹²⁵I) labeling, chemical modification (for example, biotinylation), detection or purification and then incubated with a membrane fraction of cells such as HUVEC.

The labeled CF6 is also usable in the method of identifying a CF6 agonist or a CF6 antagonist in competition with the binding of CF6 to its receptor with the use of binding assay. Standard methods for carrying out such assays are well known in the art.

(Examples of diseases)

The term "diseases associated with the excess of CF6" as used herein means diseases wherein the function of CF6 in the blood is accentuated to a level undesirable for a living body. Namely, these diseases are not always restricted to those wherein the blood CF6 level is higher than the level of normal persons. Namely, diseases associated with the shortage of PGI₂ and diseases associated with the attenuation of the cPLA₂ function fall within this category. Examples thereof include diseases associated with accentuated platelet agglutination, diseases associated with peripheral circulatory failure caused by inhibited vasodilatation, heart infarction, angina pectoris, heart failure, pulmonary hypertension, hypertension, cerebrovascular disorder, arteriosclerosis obliterans, arteriosclerosis, hyperlipemia, diabetes, bronchial disease, stomach ulcer, eclampsia of pregnancy, hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura.

The term "diseases associated with the shortage of CF6" as used herein means diseases wherein the function of CF6 in the blood is attenuated to a level undesirable for living body. Namely, these diseases are not always

restricted to those wherein the blood CF6 level is lower than the level of normal persons. Namely, diseases associated with the excess of PGI₂ and diseases associated with the accentuation of the cPLA₂ function fall within this category. Examples thereof include brain infarction, acute pancreatitis, asthma, ARDS and rheumatoid arthritis. (Provision of pharmaceutical compositions for treatment)

According to the present invention, the pharmaceutical composition for the treatment of the above-described diseases associated with the excess or shortage of the blood CF6 level can be provided.

In case where the CF6 activity is in excess, the CF6 activity can be lowered by administering antagonists or CF6 secretion inhibitory substances in an amount efficacious in inhibiting the action of CF6 on ligands, substrates, receptors, enzymes, etc. These antagonists or CF6 secretion inhibitory substances can be obtained by the screening method as described above.

To treat abnormal conditions associated with the shortage of CF6, on the other hand, the CF6 activity can be elevated by administering agonists or CF6 secretion accelerating substances in an amount efficacious in promoting the action of CF6 on ligands, substrates, receptors, enzymes, etc. These agonists or CF6 secretion accelerating substances can be obtained by the screening method as described above.

(Method of producing preparations)

Medicinal preparations of the CF6 secretion

accelerating substances, CF6 secretion inhibitory substances, CF6 agonists or CF6 antagonists obtained by using the screening method according to the present invention can be produced by conventional methods. For
5 example, these preparations may be used in the dosage form of solid preparations such as optionally coated tablets, capsules, granules or fine powder; injections, eye drops or nasal drops obtained by dissolving in water or other pharmaceutically acceptable liquid vehicles; or external
10 preparations such as ointments or patches.

These preparations can be produced in a conventional manner with the use of additives commonly employed in the art. In case of the production of solid preparations for oral administration such as tablets, capsules, granules and
15 fine powder, use can be made as additives of: (1) fillers such as lactose, starch and crystalline cellulose; (2) binders such as hydroxypropylcellulose and polyvinyl pyrrolidone; (3) disintegrating agents such as starch and croscarmellose sodium; (4) plasticizers such as macrogol
20 and triethyl citrate; (5) lubricants such as magnesium stearate and talc; (6) coating bases such as hydroxypropylmethylcellulose and Eudragit; (7) taste correctives such as sucrose and mannitol; and odor correctives, coloring agents, etc.

25 In case of the production of injections, eye drops and nasal drops, use can be made as additives of: (1) tonicity agents such as sodium chloride, D-mannitol and D-sorbitol; (2) pH controlling agents such as hydrochloric

acid and citric acid; (3) buffers such as sodium citrate, sodium acetate and boric acid; (4) soothing agents such as procaine hydrochloride; and stabilizers, surfactants, etc. Considering the stability, etc. of the active ingredient, these preparations may be either dissolved or suspended just before using or in the form of solutions.

In case of the production of external preparations such as ointments and patches, use can be made as additives of: (1) bases such as liquid paraffin, vaseline and hydrophilic ointment; (2) emulsifiers such as polysorbate 80 and tragacanth; (3) preservatives such as sodium benzoate and propyl parahydroxybenzoate; (4) soothing agents such as procaine hydrochloride; and stabilizers, surfactants, etc.

The preparations thus prepared are packaged in an appropriate manner depending on the dosage form. Examples of the package styles include bottling, folding, PTP-packaging, ampul-filling and vialling. The preparations thus obtained can be administered to, for example, mammals such as rat, rabbit, sheep, pig, cow, cat, dog, monkey, humans, etc. The administration dose varies depending on the indication, symptom, administration route, etc. In case of a human adult weighing 60 kg, the daily dose generally ranges from about 0.01 to 300 mg, preferably from about 0.1 to 100 mg. In case of other animals, the administration dose may be calculated by converting the bodyweight into a corresponding fraction of 60 kg.

(Diagnostic methods)

The present invention also provides methods of diagnosing diseases associated with an increase or a decrease in the CF6 level which comprises measuring the CF6 level in collected blood samples. As described above, the
5 CF6 level can be measured by the RIA method or the ELISA method with the use of antibody.

(CF6 gene or mRNA)

According to the present invention, it is possible to use a CF6 gene or mRNA usable as a diagnostic or a
10 diagnostic aid for the underexpression or hyperexpression of CF6, changes in the localization of CF6 or diseases caused thereby.

Individuals having a mutated CF6 gene can be detected by various methods for measuring DNA sequences. The
15 nucleic acids to be employed in the diagnosis can be obtained from the sample cells present in, for example, blood, urine, saliva, biopsy specimens or autopsy specimens. Genomic DNA may be either employed as such or amplified prior to the analysis by the PCR method or the like. mRNA
20 or cDNA may be used in the same manner. Thus, a deletion or insertion can be detected based on the difference in the amplification product size from the normal genotype. A point mutation can be identified by hybridizing the amplified DNA with CF6 DNA having the normal DNA nucleotide
25 sequence. Completely matched sequences can be distinguished from sequences of mismatched double strands by digesting with RNase or by measuring difference in melting temperature. Also, a difference of DNA sequences can be

detected based on a change in the mobility in gel when a DNA fragment is electrophoresed alone or together with a denaturing substance. It is also possible to detect the difference by directly determining the DNA sequence. A

5 change in the sequence at a specific position can be clarified by a nuclease protective assay, for example, protection with RNase and S1 or by the chemical cutting method. Alternatively, it is also possible to construct an array of oligonucleotide probes containing the CF6
10 nucleotide sequence or fragments thereof and carry out, for example, an effective screening of a genetic mutation. This array method, which has been widely known, is applicable over a broad scope and is usable in studying various problems in the field of molecular genetics such as gene
15 expression, genetic linkage and genetic change.

[Examples]

Now, the invention will be illustrated in greater detail by reference to the following Examples. However, it is to be understood that these Examples merely provide
20 modes for carrying out the present invention and the invention is not to be construed as being limited thereto. Unless otherwise noted, technical terms and abbreviations are employed in these Examples in accordance with the common usage in the art. The plasmids, *E. coli* and
25 fundamental expression procedures employed in common in these Examples will be illustrated below for reference. To confirm and illustrate the prior art providing the basis of the present invention and the related art of the present

invention, Referential Examples will be also given.

(Plasmid)

pG97S4DhCT[G] is a plasmid capable of expressing a chimeric protein wherein hCT[G] (a peptide having Gly added to the C-terminus at the 32-position of human calcitonin) is bonded via a sequence Glu-Phe-Glu to a peptide consisting of the amino acids from the N-terminus to the 97-position of β -galactosidase (called β Gal-97S4D represented by SEQ ID NO:5 wherein Cys at the 76-position is substituted by Ser and Glu at the 40-, 41- 71- and 75-positions are substituted by Asp) by an *E. coli* lactose operon promoter. A transformant obtained by using this plasmid can be selected depending on resistance to tetracycline. A chimeric protein with β Gal-97S4D can be expressed by transferring an EcoRI-SalI DNA fragment consisting of the DNA domain encoding a target peptide and the coding frame. An *E. coli* W3110 strain containing this plasmid was named *Escherichia coli* SBM323 and deposited with National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology under the accession number FERMBP-3503 on August 8, 1991.

pGP#19RI is a plasmid wherein a gene encoding the chimeric protein β Gal-97S4D/hCT[G] of pG97S4DhCT[G] is substituted by another gene encoding a chimeric protein β Gal-139S(SEQ ID NO:4)/hProPTH. A chimeric protein with β Gal-139S can be expressed by transferring an EcoRI-SalI DNA fragment consisting of the DNA domain encoding a target peptide and the coding frame. This plasmid pGP#19RI can be

constructed by inserting the EcoRI site into the DNA domain encoding the bond between the protective peptide β Gal-139S and ProPTH of a plasmid pGP#19 (unexamined published Japanese application (Kokai) No.9-296000; Fig. 4).

5 A plasmid pCRII was purchased from Invitrogen Co. in the state of allowing direct linkage to DNA fragments having been amplified by the PCR method with the use of Taq DNA polymerase. A transformant obtained by using this plasmid pCRII can be selected depending on resistance to
10 ampicillin.
 (*E. coli* and media)

 The *E. coli* JM109 strain was purchased from Toyobo Co., Ltd. and employed in preparing the plasmids and expressing the chimeric proteins.

15 The media employed were an LB medium (0.5% (w/v) of yeast extract, 1% (w/v) of trypton, 0.5% (w/v) of NaCl) and an SB medium (2% (w/v) of yeast extract, 1% (w/v) of trypton, 0.5% (v/v) of glycerol, 1.2% (w/v) of K_2HPO_4 , 0.3% (w/v) of KH_2PO_4).

20 (Fundamental experimental procedures)

 Unless otherwise stated in particular, the following experimental procedures were employed in Examples. DNA nucleotide sequences were determined by using the dideoxy method with a DNA Sequencer 373 (Applied Biosystems). To
25 cleave DNA with restriction enzymes, the reaction was performed for 1 hour using the enzymes in an amount 3 to 10 times more than the amount specified by the manufacturer. Plasmid structures were analyzed in 20 μ L of a liquid

reaction mixture by using 0.5 to 1 μ g of DNA. Preparation of DNA was carried out in 50 to 100 μ L of a liquid reaction mixture by using 3 to 10 μ g of DNA. The reaction conditions (temperature, buffer, etc.) were set up in accordance with the manufacturer's instruction. The obtained DNA fragments were separated by agarose gel electrophoresis and subjected to analysis or preparation.

Samples for the agarose gel electrophoresis were prepared by adding 1.5 times by volume as much a colorant solution (15% (w/v) aqueous solution of Ficoll containing 0.25% (w/v) of Bromophenol Blue) to liquid reaction mixtures. As the buffer for the agarose gel electrophoresis, use was made of a TAE buffer (10 mM of tris, 20 mM of acetic acid, 2 mM of EDTA·Na, pH 6.8). In analyzing the plasmid structures and preparing the DNA fragments, migration was carried out by using Mupid-2 (COSMO BIO) at 100 V for 1 hour. The gel was stained with an aqueous solution of ethidium bromide (0.5 μ g/ml) for 20 minutes and then irradiated with ultraviolet light to detect DNA bands. The concentration of the agarose gel was adjusted to 1.0, 1.5 and 2.0% (w/v) depending on the size of the DNA fragment to be fractionated. DNA in the agarose gel was extracted from the gel by using SUPREC-01 (Takara Shuzo Co., Ltd.). DNA solutions were treated with phenol and then subjected to precipitation from ethanol. Ligation reactions were carried out by using 0.05 to 1 μ g of DNA fragments with the use of a TAKARA Ligation Kit (Takara Shuzo Co., Ltd.).

E. coli was transformed by the calcium chloride method, wherein competent cells of JM109 strain were employed, and transformants were selected depending on the resistance to drugs (ampicillin or tetracycline).

5 The SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed in accordance with Laemmli's method (Laemmli et al. Nature 227, 680-685, 1970). Namely, 1/4 times by volume as much 4xSDS sample buffer (375 mM of tris hydrochloride (pH 6.8), 30% (v/v) of glycerol, 7% (w/v) of SDS, 15% (v/v) of 2-mercaptoethanol and 0.1% (w/v) of Bromophenol Blue) was added to a sample and heated to 95°C for 5 minutes. Then 10 µL of the reaction mixture was subjected to SDS-polyacrylamide gel (55 mm x 85 mm x 1 mm, Tefco) and electrophoresed at 20 mA for 80 minutes. After 10 the completion of the electrophoresis, the gel was stained 15 with a staining solution (10% (v/v) of acetic acid, 40% (v/v) of methanol and 0.25% (w/v) of Coomassie Brilliant Blue R250).

20 In the analysis and purification of peptides by HPLC with the use of a C18 column, use was made of an HPLC LC10A (Shimadzu Corporation) connected to an YMC00DS-A302 column (diameter: 4.6 mm x 150 mm), an YMC-ODS-A323 column (diameter: 10 mm x 250 mm) (YMC K.K.) and an Inertsil ODS-2 C18 column (diameter: 4.6 mm x 250 mm) (GL Science K.K.). 25 Development was carried out under concentration gradient of a solution A (0.1% (v/v) trifluoroacetic acid (TFA)) and a solution B (0.092% (v/v) TFA/80% (v/v) acetonitrile, or 0.092% (v/v) TFA/60% (v/v) acetonitrile) at a flow rate of

1 ml/min or 2.5 ml/min. The peak area of the absorbance at 210 nm was measured.

DNA oligomers were synthesized by Greiner Japan or Lifetech Oriental.

5 The activity of inhibiting the PGI_2 production was evaluated by measuring the stable metabolite of PGI_2 (6-keto- $\text{PGF}_{1\alpha}$) secreted into the medium of smooth muscle cells collected from SHR mesenteric artery or human umbilical venal endothelial cells (HUVEC). Namely, the smooth muscle
10 cells collected from SHR mesenteric artery were subcultured in a Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) of fetal calf serum until these cells attained the confluent state. HUVEC were proliferated in a synthetic medium (HuMedia, Kurabo Industries) containing 2% (v/v) of
15 fetal calf serum, 10 ng/ml of EGF, 5 ng/ml of bFGF, 1 $\mu\text{g/ml}$ of hydrocortisone and 10 $\mu\text{g/ml}$ of heparin. The smooth muscle cells collected from SHR mesenteric artery or HUVEC were incubated for 30 minutes in the DMEM medium containing the sample to be analyzed and 6-keto- $\text{PGF}_{1\alpha}$ secreted into
20 the medium during the incubation period was determined.

Blood pressure was measured in the open manner by inserting catheters directly into the aorta of rats anesthetized with 75 mg/kg of ketamine and 15 mg/kg of xylazine hydrochloride. Namely, two polyethylene catheters
25 filled with physiological saline containing 20 U/ml of heparin were inserted respectively into the left carotid artery and the left femoral vein. Then the catheter inserted into the left carotid artery was connected to a

pressure detector (Carrier Amplifier AP-601G, Nihon Kohden Co.). Each sample was dissolved in 200 µL of physiological saline and then administered via the vena cava.

The instruments and reagents were each used in accordance with the manufacturer's instruction. Fundamental gene manipulation procedures were carried out by reference to Maniatitis et al., "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory, 1989). [Example 1] Preparation of CF6 by chimeric protein

10 expression method-1

Next, a method of producing CF6 by using enterokinase as an enzyme for cutting it off from a chimeric protein will be described in detail.

(Preparation of rCF6 cDNA and hCF6 cDNA)

15 rCF6 cDNA was obtained by the PCR method with the use of rat liver cDNA (Quick Clone cDNA Library, Clontech) as a template and rCF6-01 (SEQ ID NO:6) and rCF6-02 (SEQ ID NO:7) as primers. Namely, by using KOD (Toyobo Co., Ltd.) as a thermostable DNA polymerase, a liquid reaction mixture 20 comprising 1 µL of the rat liver cDNA library, 5 µL of 10 x reaction buffer, 7.5 µL of 2 mM of dNTP mix, 0.5 µL of rCF6-01, 0.5 µL of rCF6-02 and 35 µL of sterilized water was subjected to the reaction for 30 cycles with each cycle consisting of 30 seconds at 96°C, 1 minute at 56°C and 40 25 seconds at 74°C. Thus rCF6 cDNA was obtained. Similarly, hCF6 cDNA was obtained by the PCR method with the use of human kidney cDNA (Quick Clone cDNA Library, Clontech) as a template and hCF6-01 (SEQ ID NO:8) and hCF6-02 (SEQ ID

NO:9) as primers. The reaction conditions were as in the case of the rat.

SEQ ID NO:6 5'- ATGACTGTTTCAGAGGATCTTCAG -3'

SEQ ID NO:7 5'- GTCGACTCAGGACTGGGGTTTGTTCGAG -3'

5 SEQ ID NO:8 5'- ATGATTCTTCAGAGGCTCTTCAG -3'

SEQ ID NO:9 5'- GTCGACTCAGGCCTGGGGTTTTTCGATG -3'.

The rCF6 cDNA and hCF6 cDNA thus obtained were each electrophoresed on a 1.5% (w/v) agarose gel, collected by using SUPREC-01 (Takara Shuzo Co., Ltd.) and then used as a
10 template for constructing a chimeric protein gene from which rCF6 or hCF6 could be cut off respectively with enterokinase.

(Preparation of rCF6 and hCF6 having enterokinase recognition sequence added thereto)

15 To construct the chimeric protein gene from which rCF6 or hCF6 could be cut off with enterokinase, a DNA oligomer having a gene sequence encoding Asp-Asp-Asp-Asp-Lys and a restriction enzyme EcoRI recognition site in the 5' side of a gene encoding the N-terminal side of rCF6 or
20 hCF6 was synthesized (rCF6E: SEQ ID NO:10 and hCF6E: SEQ ID NO:11).

SEQ ID NO:10

5'- GAATTCGACGATGACGATAAGAATAAGGAACTTGATCCTGTACAG -3'

SEQ ID NO:11

25 5'-GAATTCGACGATGACGATAAGAATAAGGAACTTGATCCTATACAGA -3'.

The DNA sequence of SEQ ID NO:10 was transferred by the PCR method with the use of the rCF6 cDNA as a template and rCF6E (SEQ ID NO:10) and rCH6-02 (SEQ ID NO:7) as

primers. As the thermostable DNA polymerase in PCR, use was made of Taq DNA Polymerase (Pharmacia). Namely, a liquid reaction mixture comprising 1 μ L of rCF6 cDNA, 5 μ L of 10 x reaction buffer, 8 μ L of 1.25 mM of dNTP mix, 0.5 μ L of rCF6-01, 0.5 μ L of rCF6-02 and 25 μ L of sterilized water was subjected to the reaction for 8 cycles with each cycle consisting of 1 minutes at 96°C, 1 minute at 60°C and 30 seconds at 72°C. Thus rCF6 cDNA having the enterokinase recognition sequence and the restriction enzyme EcoRI

10 recognition sequence in the 5'-side was obtained. The DNA fragment thus amplified was cloned into pCRII (pCRrCF6EK) and the nucleotide sequence was confirmed. Similarly, the DNA sequence of SEQ ID NO:11 was transferred by the PCR method with the use of the hCF6 cDNA as a template and hCF6E (SEQ ID NO:11) and hCF6-02 (SEQ ID NO:9) as primers. The reaction conditions were as in the case of the rat. The DNA fragment thus amplified was cloned into pCRII (pCRhCF6EK) and the nucleotide sequence was confirmed. (Construction of chimeric protein-expression vectors)

20 The plasmids pCRrCF6EK and pCRhCF6EK were cut with restriction enzymes EcoRI and SalI to give EcoRI-SalI DNA fragments rCF6EK and hCF6EK each consisting of 0.3 kb. These DNA fragments have DNA nucleotide sequences encoding proteins containing rCF6 or hCF6 having an enterokinase recognition sequence Asp-Asp-Asp-Asp-Lys (SEQ ID NO:3) bonded to the N-terminus and a restriction enzyme EcoRI site in the upstream thereof and another restriction enzyme SalI site in the downstream thereof. The plasmid pGP#19RI

was cut with the restriction enzymes EcoRI and SalI and then a DNA fragment of about 3.5 kb containing the vector part was prepared. This DNA fragment was ligated to rCF6EK or hCF6KE, thereby giving plasmids pG139SrCF6EK and pG139ShCF6EK respectively.

(Expression and purification of chimeric proteins)

The *E. coli* JM109 strain was transformed by using the plasmids pG139SrCF6EK and pG139ShCF6EK, thereby giving JM109[pG139SrCF6EK] and JM109[pG139ShCF6EK] respectively. JM109[pG139SrCF6EK] and JM109[pG139ShCF6EK] were each cultured in an SB medium containing 10 µg/mL of tetracycline under aeration and agitation at 37°C with the use of a 3 L jar fermenter. 8 hours thereafter, IPTG was added to give a final concentration of 1 mM and the culture was continued overnight. 3 L of the liquid culture was centrifuged at 6,000 rpm at 4°C for 10 minutes by using a centrifuge 20PR-52D (Hitachi, Ltd.) and then the cells were collected. The cells were suspended in 20 mM tris hydrochloride (pH 7.0) containing 0.5% (w/v) of Triton X-100, 1 mM EDTA and disrupted by ultrasonication. Then the disrupted cells were centrifuged at 6,000 rpm at 4°C for 15 minutes by using a centrifuge 05PR-22 (Hitachi, Ltd.). Thus, the chimeric protein was collected in the precipitate fraction as an inclusion body. The dissolution and centrifugation of the precipitate fraction were repeated thrice to thereby give a partially purified chimeric protein. The hCF6 chimeric protein was prepared in the same manner.

(Cutting off of hCF6 and rCF6 from chimeric proteins and purification)

As a method of cutting off rCF6 from a chimeric protein, the method with the use of factor Xa as a cutting enzyme may be cited (Referential Example 2). To solubilize the chimeric protein and treat it with the factor Xa in this method, it is necessary to use at least 2 M of urea. At this urea concentration, the activity of the factor Xa is lowered to 1/10. Therefore, it is necessary in this case to use a large amount of the factor Xa, i.e., 1/60 times as much as the chimeric protein. In addition, the factor Xa shows only a low substrate specificity in the presence of urea. As a result, the cutting efficiency from the chimeric protein was as low as about 8%.

Therefore, attempts were made to elevate the CF6 cutting efficiency by using enterokinase as a cutting enzyme. First, 4 mg of the crude rCF6 chimeric protein was solubilized in 1.75 mL of a 10 M urea solution. Then, the obtained solution was centrifuged to thereby eliminate insoluble matters. To the supernatant, 0.5 mL of 10 x EK buffer (200 mM of tris hydrochloride, (pH 8.0), 500 mM of NaCl, 20 mM of CaCl_2) and 30 U (about 3.4 μg , corresponding to about 1/1200 of the substrate) of enterokinase (Stratagene) were added and the total volume of the mixture was adjusted to 5 mL with distilled water. After reacting at 22°C overnight, the liquid reaction mixture was poured into SepPak 18 (Waters) to thereby adsorb the peptides. The peptides thus adsorbed were eluted with an 80% (v/v)

acetonitrile solution containing 0.1% (v/v) of TFA. The eluate was freeze-dried and then dissolved in 0.1% (v/v) TFA. A 1/10 portion thereof was subjected to HPLC connected to YMC-ODS-A302 (diameter: 4.6 mm x 150 mm).

5 Then a peak eluted about 14.2 minutes after loading the sample was collected as an rCF6 fraction (Fig. 2). The same procedure was repeated 10 times and the fractions thus eluted were combined, thereby giving partially purified rCF6.

10 This partially purified rCF6 fraction was freeze-dried, then dissolved in 0.1% TFA and then purified by HPLC connected to YMC-ODS-AM-323 (Fig. 3, %B:40-43/18 min, flow rate: 2.5 ml/min). The peak eluted after about 14.2 minutes was collected and freeze-dried to give an rCF6
15 specimen. The structure of rCF6 was confirmed based on the facts that it showed a single peak in mass spectrometry with the use of a mass spectrometer MALDI-TOFMS (Voyager Elite: Nippon PerSeptive), that its molecular weight roughly agreed with the theoretical value, and that the 10
20 amino acids at the N-terminus were identical with those of rCF6, when analyzed by using an amino acid sequence analyzer PPSQ-10 (Shimadzu Corporation).

hCF6 was cut off from the chimeric protein and purified as in the case of rCF6. The hCF6 was eluted after
25 about 18.0 minutes in the first HPLC (Fig. 14). In the chromatographic re-purification, it was eluted after about 10.5 minutes (Fig. 5, elution concentration gradient, %B:45-48/18 min). The structure was confirmed as

in the case of rCF6.

About 210 µg and about 290 µg of hCF6 and rCF6 were respectively obtained from 400 mg portions of the chimeric proteins and the cutting efficiencies were about 15% and
5 about 20% respectively. Namely, the cutting efficiencies were elevated 2 to 2.5-fold, compared with the case where the factor Xa was employed as the cutting enzyme (yield: 8%).

[Example 2] Assay of CF6 in rat blood

10 (Preparation of antirat CF6 rabbit antibody (anti-rCF6 antibody))

A peptide consisting of 20 amino acids in total, wherein cysteine was added to the N-terminus of the 19 C-terminal amino acids of rCF6 (SEQ ID NO:2) (i.e.,
15 (CFPTFNFEDPKFEVLDPQS : C-rCF6 (58-76):SEQ ID NO:12) , was synthesized by the Fmoc solid phase method and purified by HPLC with the use of a C18 column. Then an antigen for immunization was prepared by bonding keyhole limpet hemocyanin (KLH) to the peptide thus synthesized via the
20 N-terminal cysteine.

As animals, two female New Zealand white rabbits were employed. At the priming, the antigen emulsified with complete Freund's adjuvant (CFA) was subcutaneously administered at 4 positions of the dorsal
25 part of the rabbits. 2, 6 and 8 weeks thereafter, the antigen emulsified with incomplete Freund's adjuvant (IFA) was administered in the same manner to thereby immunize the animals. Then all of the blood samples of the animals were

collected and thus antisera were obtained. The antibody
titers of the antisera were measured by ELISA with the
immobilization of the antigen. The antibody titers of the
rabbit sera before the immunization were each 50 or less,
5 while the antibody titers of the antisera of the immunized
rabbits were 59,600 and 81,400 respectively. The antibody
was purified from the antisera by the immunoaffinity
chromatography using an antigen peptide as the affinity
ligand (Fig. 6). The antibody thus purified was
10 concentrated, dialyzed against a PBS solution containing
0.1% (w/v) of NaN_3 and then stored at -80°C until it was
used.

(Setting up of method of measuring rCF6 level)

A peptide consisting of 20 amino acid in total,
15 wherein tyrosine was added to the N-terminus of the 19
C-terminal amino acids of rCF6 (SEQ ID NO:2) (i.e.,
(YFPTFNFDPKFEVLDPQS:Y-rCF6 (58-76):SEQ ID NO:13) , was
synthesized by the Fmoc solid phase method and purified by
HPLC with the use of a C18 column. Y-rCF6(58-76) was
20 labeled with radioactive iodine (^{125}I -labeling) by the
lactoperoxidase method. The ^{125}I -labeled Y-rCF6(58-76) was
purified by HPLC with the use of a C18 column and then
bovine serum albumin was added to give a final
concentration of 0.1% (w/v). Then it was stored at -80°C
25 until it was used.

As the reaction system, use was made of an
antagonistic system wherein the reaction between the
 ^{125}I -labeled antigen and the antibody was quantified by

inhibiting by the antigen in a sample. Namely, 100 μ L of a sample containing 0.25 to 32 ng of rCF6 was mixed with 100 μ L of the rCF6 antibody (diluted 2500-fold) and reacted for 12 hours. Then 100 μ L (20,000 cpm) of the 125 I-labeled Y-rCF6(58-76) was added thereto and the mixture was further reacted overnight. Next, 1 mL of antirabbit IgG goat serum (Japan Immunoresearch Laboratories Co., Ltd.) diluted 60-fold with RIA buffer (50 mM of Na_2HPO_4 , 80 mM of NaCl, 25 mM of EDTA \cdot 2Na, 0.05% (w/v) of NaN_3 , 0.5% (w/v) of bovine serum albumin, pH 7.4) containing 100 μ L of 200 μ g/mL rabbit IgG and 10% (w/v) of polyethylene glycol 6000 was added and the resultant mixture was allowed to stand for 1 hour. Then it was centrifuged at 3,000 rpm for 30 minutes and the supernatant was eliminated. The 125 I level in the supernatant was measured with a gamma counter. These reactions were all carried out at 4°C. As a result, dose-dependent radioactivity was observed in the precipitate within the range of 0.25 to 16 ng/100 μ L. Thus, a method of measuring the rCF6 level in a sample could be set up.

20 (Assay of rCF6 in rat blood)

By using the assay method as described above, measurement was made of rCF6 in the blood of male spontaneously hypertensive rats (SHR) and rats, 4 weeks of age, with normal blood pressure (WKY), and SHR and WKY of 16 weeks of age.

Each group had 5 animals. The blood of each animal was collected by using a cylinder containing a 1% (w/v) EDTA \cdot 2Na solution in 1/10 dose. The blood thus obtained

was centrifuged under cooling to extract the plasma. The plasma was stored at -80°C until the assay. 0.2 ml of 2 N hydrochloric acid was added to 0.8 ml of the plasma and the mixture was allowed to stand at 4°C for 10 minutes. Next,
5 it was centrifuged at 4°C at 10,000 rpm for 30 minutes. The supernatant was added to a SepPak C18 column and washed with 5 ml portions of 0.1% (v/v) TFA thrice. After eluting from the column with 2 ml of a 60% (v/v) acetonitrile solution containing 0.1% (v/v) of TFA, the solvent was
10 removed in vacuo and the residue was dissolved in 0.2 to 0.4 ml of RIA buffer to give a sample for RIA which was then used in the assay method with the use of the anti-rCF6 antibody as described above.

As the results of the assay of rCF6 in the plasma of
15 the rats of each group, every group showed a plasma rCF6 level exceeding 200 pg/mL. At the age of 4 weeks, no difference was observed between the plasma rCF6 levels of SHR and WKY. At the age of 16 weeks, the plasma rCF6 level of SHR was significantly increased and thus higher than
20 that of WKY ($p < 0.05$) (Table 1).

Thus, it is clarified for the first time that rCF6 is present in rat blood. It is also found out that the blood rCF6 level increases with aging (i.e., the onset of hypertension) in the spontaneously hypertensive model (SHR)
25 suffering from the shortage of PGI_2 . That is to say, it is clarified that the onset of hypertension caused by the shortage of PGI_2 correlates to the blood CF6 level.

[Table 1]

Table 1: Plasma rCF6 level (pg/ml)

Age	WKY	SHR
4 weeks	414±123	216±108
16 weeks	294±126	2310±813

Expressed in mean ± standard error.

(Molecules in anti-rCF6 antibody-reactive fraction of RIA sample by HPLC)

5 Anti-rCF6 antibody was prepared by using an rCF6
fragment (58-76) as an antigen. Thus, the amount of CF6
measured in the above-described RIA system included the
peptides containing the C-terminal fragment originating in
rCF6. The RIA sample was fractionated by HPLC and the
10 reactivity of anti-rCF6 antibody of each fraction was
examined. The RIA sample was adsorbed by SepPak C18 and
eluted with a 60% (v/v) acetonitrile solution containing
0.1% (v/v) of TFA. The sample thus eluted was freeze-dried,
dissolved in 0.1% (v/v) TFA and separated and fractionated
15 by HPLC connected to an Inertsil ODS-2 C18 column
(diameter:4.6 mm x 250 mm, GL Science K.K.) by washing with
a 20% (v/v) acetonitrile solution containing 0.1% (v/v) of
TFA for 5 minutes or longer and then eluting under linear
gradient of acetonitrile (20-60%) for 30 minutes. Thus, it
20 was confirmed that 90% or more of the antibody reactivity
was eluted in the same fraction as rCF6 in the assay system
with the use of the anti-rCF6 antibody as described above.

These results indicate that 90% or more of molecules
reactive to the anti-rCF6 antibody present in the blood are
25 not peptides containing the C-terminal fragment originating

in rCF6 but rCF6 per se.

[Example 3] Preparation of anti-hCF6 rabbit antibody (anti-hCF6 antibody) and construction of hCF6 assay system

Antisera against hCF6 were obtained as in Example 2
5 but using as an antigen for immunization a peptide
consisting of 19 amino acid wherein cysteine was added to
the N-terminus of the N-terminal amino acids (10-27) of
hCF6 (SEQ ID NO:1) (i.e., CLFVDKIREYKSKRQTSGG:C-hCF6 (10-
27), SEQ ID NO:14) bonded to KLH. The antibody titers of
10 the immunized rabbits were 123,000 and 121,300. The anti-
hCF6 antibody was purified as in Example 2. The hCF6 assay
system was constructed as in the rCF6 assay system but
using a peptide consisting of the N-terminal amino acids
(10-27) of hCF6 (SEQ ID NO:2) (LFVDKIREYKSKRQTSGG:hCF6(10-
15 27), SEQ ID NO:15) as the ¹²⁵I-labeled peptide and hCF6 as
the standard.

[Example 4] Effects of anti-rCF6 antibody on rat blood
pressure

(Effects of anti-rCF6 antibody on rat blood pressure)

20 SHR and WKY each 16 weeks of age were anesthetized
and the anti-rCF6 antibody (0.3, 1.0 and 3.0 µg/kg) was
administered by bolus injection into the femoral vein.
Then the arterial blood pressure was measured in the open
manner from the carotid artery. As a result, the average
25 arterial blood pressure of SHR was lowered in the dose-
dependent manner by administering the anti-rCF6 antibody
(Figs. 7 and 8). In WKY, on the other hand, no significant
change in the blood pressure was observed due to the

administration of the anti-rCF6 antibody (Fig. 8). The administration of a nonspecific rabbit antibody (IgG: 10 µg/kg) did not affect the arterial blood pressures of SHR and WKY.

- 5 (Effects of the preadministration of anti-rCF6 antibody on decrease in rat blood pressure due to bradykinin)

Effects of the anti-rCF6 antibody on the effect of bradykinin of lowering rat blood pressure were examined. SHR and WKY each 16 weeks of age were anesthetized and
10 bradykinin (0.3, 1.0 and 3.0 µg/kg) was cumulatively administered by bolus injection into the femoral vein. Then the arterial blood pressure was measured in the open manner from the carotid artery. Next, the anti-rCF6 antibody (3.0 µg/kg) was administered by bolus injection
15 into the femoral vein and then bradykinin was administered in the same manner. The blood pressure reaction was observed.

As a result, the blood pressures of SHR and WKY were dose-dependently lowered by the administration of
20 bradykinin (Figs. 9-1 and 10). In SHR, the administration of the anti-rCF6 antibody potentiated the effect of bradykinin of lowering blood pressure (Figs. 9-2 and 10), while no effect was observed in WKY (Fig. 10).

As described above, the anti-rCF6 antibody exerted a
25 hypotensive effect exclusively in the hypertensive state with an increased rCF6 level. Thus, it is indicated that the inhibition of CF6 is efficacious in ameliorating symptoms of diseases caused by the excess of CF6 in the

blood.

[Example 5] Effects of the administration of rCF6 on rat blood pressure

Effects of rCF6 on rat blood pressure were examined.

5 SHR or WKY 15 weeks of age were anesthetized and rCF6 (0.3, 1.0 and 3.0 $\mu\text{g/kg}$) was administered by bolus injection into the femoral vein. Then the arterial blood pressure was measured in an open manner from the carotid artery. As a result, the average arterial blood pressures of SHR and WKY
10 were dose-dependently increased by the administration of rCF6. A remarkable effect was observed in SHR compared with WKY (Figs. 11 and 22).

Namely, a more remarkable effect of CF6 was observed on SHR having a higher blood CF6 level. This fact
15 indicates that SHR is more sensitive to the effect of CF6.

Considering this result and the hypotensive effect of the anti-rCF6 antibody on SHR observed in Example 4, it is expected that CF6 agonists and CF6 secretion inhibitory drugs would be usable as radical remedies for hypertension
20 which is a disease associated with the excess of CF6 in the blood. Namely, it is indicated that the control of blood CF6 level is efficacious as a method of treating diseases associated with abnormal CF6 level in the blood.

[Example 6] Plasma hCF6 levels of normal subjects and
25 patients with acute heart infarction

Blood samples of 6 normal subjects and 18 patients with acute heart infarction were collected from the vein with the use of a cylinder containing a 1% (w/v) EDTA·2Na

5 solution in 1/10 dose. All of the patients with acute heart infarction had been successfully treated by percutaneous transluminal coronary angioplasty (PTCA). The blood samples were collected on the day of the onset and the days 2, 3, 5, 7 and 14. Each blood sample thus obtained was centrifuged under cooling to extract the plasma. The plasma was stored at -80°C until assaying.

10 A sample for assaying hCF6 was prepared from the plasma in the following manner. Namely, 0.2 ml of 2 N hydrochloric acid was added to 0.8 ml of the plasma and the mixture was allowed to stand at 4°C for 10 minutes. Next, it was centrifuged at 4°C at 10,000 rpm for 30 minutes. The supernatant was added to a SepPak C18 column and washed with 5 ml portions of 0.1% (v/v) TFA thrice. After eluting from the column with 2 ml of a 60% (v/v) acetonitrile solution containing 0.1% (v/v) of TFA, the solvent was removed in vacuo and the residue was dissolved in 0.2 to 0.4 ml of RIA buffer to give a sample for RIA.

20 The hCF6 level was measured as in the case of rCF6 (Example 3) but using recombinant hCF6 as a standard and the anti-hCF6 antibody.

25 CF6 was detected at the concentration of 15.2 ± 0.5 ng/ml (mean \pm standard error) in the normal subject plasma. It is thus confirmed that CF6, which is a protein present in the mitochondrial inner membrane, is present not only in rat blood but also in human blood (Fig. 23). The CF6 level in the plasma of the patients with acute heart infarction showed a peak on the day of the onset or the day 2 and a

high plasma CF6 level of 22.1 ± 1.3 ng/ml was observed (Fig. 23). The plasma CF6 levels on the day 1 and day 2 were respectively 20.2 ± 1.4 ng/ml and 17.7 ± 1.5 ng/ml which were significantly higher than the concentration on day 14 (13.7 \pm 0.7 ng/ml) (Fig. 24: Fisher's PLSD: $p < 0.001$ and $p = 0.01$). The plasma CF6 levels on the 7 and day 14 were respectively 13.7 ± 0.6 ng/ml and 13.7 ± 0.7 ng/ml, i.e., lowered to the level of the normal subjects.

Thus, it is clarified that the blood CF6 level increases in patients with acute heart infarction. It is therefore suggested that the measurement of the CF6 level might be useful as a diagnostic method for diseases associated with changes in the CF6 level such as acute heart infarction.

[Example 7] Release of CF6 into HUVEC culture medium

HUVEC in the confluent state were cultured in a fetal calf serum-free 199 medium in a Petri dish (diameter: 10 cm). After 24, 48 and 72 hours, the culture medium was sampled ($n=4$ at each point) and hCF6 was quantitated. The culture medium was treated as in Example 6 and the hCF6 level was measured. Thus, it was found out that the CF6 level in the culture medium increased with the passage of time, thus indicating that hCF6 was released from HUVEC (Fig. 25).

Accordingly, CF6 secretion accelerating substances or CF6 secretion inhibitory substances can be obtained by adding candidate compounds to a liquid culture of HUVEC and comparing the amount of CF6 secreted into the culture with

a control.

[Referential Example 1] Identification of prostacyclin
production inhibitor

A prostacyclin production inhibitor was identified as
5 CF6 by the following method.

The hearts (152 g) of 187 SHR 16 to 20 weeks of age
were allowed to stand in boiling water for 10 minutes.
Then peptides were extracted therefrom with 1 N acetic acid.
The extract was fractionated in 0.5 ml portions by using
10 Sephadex G-25 (1.5 x 30 cm) (flow rate: 3 ml/min, Fig. 12).
The active fractions (19-21) were separated by HPLC
connected to an Inertsil ODS-2 C18 (diameter: 4.6 mm x 250
mm) column (GL Science K.K.). After washing with a 20%
(v/v) acetonitrile solution containing 0.1% (v/v) of
15 purified TFA for 5 minutes or longer, it was eluted with
acetonitrile under a linear gradient (20-60%) for 30
minutes (flow rate: 1 ml/min, Fig. 13). The activity was
evaluated by assaying 6-keto-PGF α secreted into the medium
obtained from smooth muscle cells originating in SHR
20 mesenteric artery. The purified peptide (Fig. 14) was
sequenced as follows. The sequence from the N-terminus to
the 39-position was identified by using an amino acid
sequence analyzer (PPSQ-10, Shimadzu Corporation) as
NKELDPVQKLFLDKIREYKA KRLASGGPVDTGPEYQQEV (1- to 39-amino
25 acids in SEQ ID NO:2, SEQ ID NO:16). By purifying the
fragment digested with AsnN by HPLC connected to YMC-ODS-
A302 (diameter: 4.6 mm x 150 mm), the subsequent sequences
were identified as DRELFLKQMYGKGEM (40- to 55-amino acids

in SEQ ID NO:2, SEQ ID NO:17), DKFPTFNFE (56- to 64-amino acids in SEQ ID NO:2, SEQ ID NO:18), DPKFEVL (65- to 71-amino acids in SEQ ID NO:2, SEQ ID NO:19) and DKPQS (72- to 76-amino acids in SEQ ID NO:2, SEQ ID NO:20). These sequences completely agreed with rCF6 (SEQ ID NO:2) which is a subunit of rat H⁺ ATP synthase .

[Referential Example 2] Preparation of CF6 by chimeric protein expression method-2

A method of producing rCF6 (SEQ ID NO:2) with the use of factor Xa as a cutting enzyme from a chimeric protein will be illustrated.

(Preparation of rCF6 DNA)

A DNA fragment containing a gene encoding a rCF6 protein having a cofactor Xa recognition sequence Ile Glu Gly Lys (SEQ ID NO:21) added to the N-terminus was obtained by PCR. A rat aorta cDNA library (Clontech) having been treated at 100°C for 1 minute was used as a template, while CF03 (SEQ ID NO:22) and CF04 (SEQ ID NO:23) were used as primers.

The DNA fragment was cloned into pCRII (pCRIIrCF6Xa) and then the DNA nucleotide sequence was determined to thereby confirm the structure.

SEQ ID NO:22 5'-GATCGAGGGACGTAATAAGGAACTTGATCCT-3'

SEQ ID NO:23 5'-GTCGACTTAGGACTGGGGTTTGTCTGA-3'.

(Construction of expression vector)

The plasmid pCRIIrCF6Xa was cut with restriction enzymes EcoRI and SalI to give a 0.3 kb EcoRI-SalI DNA fragment. This DNA fragment had a DNA nucleotide sequence

encoding a protein wherein a sequence Glu-Phe-Gly-Leu-Ile-Glu-Gly-Lys (SEQ ID NO:24) having the factor Xa recognition sequence Ile-Glu-Gly-Lys bonded to the C-terminus of a sequence Glu-Phe-Gly-Leu originating in the plasmid pCRII

5 DNA was added to the N-terminus of rCF6 and had the restriction enzyme EcoRI site in the upstream thereof and another restriction enzyme SalI site in the downstream thereof. The plasmid pG97S4DhCT[G] was cut with the restriction enzymes EcoRI and SalI and a DNA fragment of about 3.5 kb containing the vector part was prepared. Then it was ligated to the 0.3 kb EcoRI-SalI DNA fragment to thereby give a plasmid pG97SDrCF6Xa (Fig. 15).

(Expression and purification of rCF6 chimeric protein)

By using the plasmid pG97SDrCF6Xa, *E. coli* JM109 strain was transformed to give JM109[pG97SDrCF6Xa]. The obtained JM109[pG97SDrCF6Xa] was inoculated into an LB medium containing 10 µg/mL of tetracycline and cultured at 37°C for 6 hours. Then the liquid culture was inoculated into an SB medium containing 10 µg/mL of tetracycline and 5 mM of MIPTG to give a final turbidity (OD 660 nm) of 0.1 and cultured at 37°C for 16 hours.

The liquid culture was centrifuged at 6,000 rpm at 4°C for 10 minutes (20PR-52D, Hitachi, Ltd.) and the cells were collected. The cells were suspended in 20 mM tris hydrochloride (pH 8.0) containing 1 mM of EDTA·Na and disrupted with a French press (10,000 psi; twice).

The disrupted cell suspension was centrifuged at 8,000 rpm at 4°C (05PR-22, Hitachi Ltd.). The precipitate

was suspended in 30 ml of 20 mM tris hydrochloride (pH 8.0) containing 0.5% (w/w) of Triton X-100 and centrifuged at 3,000 rpm at 4°C for 15 minutes. Then, precipitate was collected. This procedure was repeated 4 times to thereby
5 give a partially purified chimeric protein.
(Cutting out of rCF6 from chimeric protein and purification)

To cut out rCF6 from the chimeric protein, 50 µg/mL of the factor Xa (Takara Shuzo Co., Ltd.) was used at a
10 concentration of 3 mg/mL of the chimeric protein and reacted in 50 mM tris hydrochloride (pH 8.0) containing 2 M of urea, 100 mM of NaCl and 1 mM solution of CaCl₂ at 37°C for 4 hours. The liquid reaction mixture was adsorbed by a SepPak C18 column and washed with 0.1% (v/v) of
15 trifluoroacetic acid. Then the peptide was collected in an 80% (v/v) acetonitrile solution containing 0.1% (v/v) of trifluoroacetic acid. The eluate was freeze-dried and then dissolved in 0.1% (v/v) TFA. rCF6 was purified by HPLC connected to YMC-ODS-A302 (%B: 35-50/30 minutes, eluted
20 after 14.5 minutes). The structure of rCF6 was confirmed as in Example 1.

In this method, however, rCF6 was cut off from the chimeric protein only at a low efficiency (about 8%). Therefore, it seems necessary to develop a more efficient
25 method of producing CF6 in order to carry out *in vivo* experiments, etc. wherein large amounts of CF6 are required.
[Referential Example 3] Inhibition of the production of prostacyclin by CF6

HUVEC were cultured at 37°C for 30 minutes in the DMEM containing 1, 10 and 100 nM of rCF6 prepared in Referential Example 2 and thus the inhibition of the PGI₂ production by CF6 was examined. The production of PGI₂ was measured with the guidance of the amount of 6-keto-PGF1 α , which is a stable metabolite of PGI₂, secreted into the medium. As a result, CF6 dose-dependently inhibited the PGI₂ production (Fig. 16). The same test was carried out in the presence of 1 μ M of bradykinin. Thus, it was found out that CF6 dose-independently inhibited the increase in the PGI₂ production due to bradykinin (Fig. 17).

Further, 10 nM of CF6 inhibited the increase in the PGI₂ production due to 2 μ g/ml of ionomycin but not the increase therein due to 10 ng/ml of arachidonic acid and 10 ng/ml of PGH₂.

PGI₂ is produced starting with a phospholipid. Namely, arachidonic acid cut off from the phospholipid by PLA₂ is converted into PGH₂ by cyclooxygenase. It is further converted by PGI₂ synthase. In this reaction, ionomycin promotes the activation of PLA₂, i.e., the cutting off of arachidonic acid from the phospholipid.

Namely, it has been found out that CF6 does not inhibit the production of PGI₂ from arachidonic acid or PGH₂ which is the downstream product in the arachidonate cascade but inhibits the PGI₂ production based on the activation of PLA₂. It is thus suggested that the PGI₂ production-inhibiting effect of CF6 is based on the inhibition of PLA₂.

[Referential Example 4] Effect of CF6 of inhibiting release of arachidonic acid

In Referential Example 3, it is indicated that the PGI₂ production-inhibiting effect of CF6 is based on the inhibition of PLA₂. On the other hand, PLA₂ releasing arachidonic acid from a fatty acid is roughly classified into Ca²⁺-dependent cytoplasmic PLA₂ (cPLA₂), Ca²⁺-dependent secretory PLA₂ (sPLA₂) and Ca²⁺-independent PLA₂ (iPLA₂) depending on the structure and biochemical properties. By using respective inhibitors, it was examined which PLA₂ was inhibited by CF6.

HUVEC were cultured for 24 hours in the presence of [³H]-labeled arachidonic acid. Then the medium was replaced by DMEM and the culture was continued in the presence of 1, 10 and 100 mM of CF6 at 37°C for an additional 30 minutes. Then the release of the [³H]-labeled arachidonic acid into the liquid culture was examined. As a result, CF6 dose-dependently inhibited the release of the [³H]-labeled arachidonic acid (Fig. 18). The same test was carried out in the presence of 1 μM of bradykinin. Thus, it was found out that CF6 dose-dependently inhibited the effect of bradykinin of promoting the release of [³H]-labeled arachidonic acid (Fig. 19).

Next, the effects of a cPLA₂ inhibitor (40 μM of AACOCF₃: arachidonic acid 3-fluoromethyl ketone), an sPLA₂ inhibitor (1 μM of OPC; oleyloxyethylphosphorylcholine) and an iPLA₂ inhibitor (1 μM of BEL: bromoenolcholine) of inhibiting the release of arachidonic acid from HUVEC were

examined. As a result, it was found out that, in HUVEC, the cPLA₂ inhibitor alone inhibited the release of arachidonic acid. Namely, the PLA₂ participating in the release of arachidonic acid is cPLA₂ (Figs. 20 and 21).

5 cPLA₂ shows a high specificity to arachidonic acid-containing phospholipids and selectively releases arachidonic acid at a Ca²⁺ concentration of μ M order. As the results of experiments with the use of mice with the hyperexpression of c PLA₂ and mice lacking cPLA₂, it has
10 been clarified that cPLA₂ plays an important role not only in the metabolism of arachidonic acid in the immediate phase but in the delayed phase of arachidonic acid which is important in inflammatory reactions. Therefore, it is expected that the inhibition of cPLA₂ induces a strong
15 inflammatory reaction (Uozumi N., et al. 390 618-622, 1997).
INDUSTRIAL APPLICABILITY

The present invention provides an antibody specific to CF6 and diagnostic methods for pathological conditions in which CF6 participates. The present invention also
20 provides a method of treating diseases such as hypertension on the basis of the inhibition of CF6 in the blood whereby normal blood pressure is not lowered. Moreover, the diagnostic methods according to the present invention contribute to the provision of methods of treating
25 pathological conditions associated with an increase or decrease in CF6 in the blood.

CLAIMS

1. A pharmaceutical composition for the prevention or the treatment of a disease associated with the excess of coupling factor-6 (CF6) in the blood which comprises a CF6 inhibitor as the active ingredient.
2. The pharmaceutical composition for the prevention or the treatment as claimed in claim 1 wherein said CF6 inhibitor is a CF6 secretion inhibitory substance or a CF6 antagonist.
3. A pharmaceutical composition for the prevention or the treatment as claimed in claim 1 or 2 wherein said disease associated with the excess of CF6 in the blood is heart infarction, angina pectoris, heart failure, pulmonary hypertension, hypertension, cerebrovascular disorder, arteriosclerosis obliterans, arteriosclerosis, hyperlipemia, diabetes, bronchial disease, stomach ulcer, eclampsia of pregnancy, hemolytic-uremic syndrome or thrombotic thrombocytopenic purpura.
4. A pharmaceutical composition for the prevention or the treatment of a disease associated with the shortage of CF6 in the blood which comprises a CF6 activator or CF6 as the active ingredient.
5. The pharmaceutical composition for the prevention or the treatment as claimed in claim 4 wherein said CF6 activator is a CF6 secretion accelerating substance or a CF6 agonist.
6. The pharmaceutical composition for the prevention or the treatment as claimed in claim 4 or 5 wherein said

disease associated with the shortage of CF6 in the blood is an inflammatory disease such as brain infarction, acute pancreatitis, asthma, ARDS or rheumatoid arthritis.

7. A pharmaceutical composition for the prevention or the treatment of a disease associated with the shortage of PGI₂ and/or a disease associated with the attenuation of the Ca²⁺-dependent cytoplasmic PLA₂ (cPLA₂) function, which comprises a CF6 inhibitor as the active ingredient.

8. The pharmaceutical composition for the prevention or the treatment as claimed in claim 7 wherein said CF6 inhibitor is a CF6 secretion inhibitory substance or a CF6 antagonist.

9. A pharmaceutical composition for the prevention or the treatment as claimed in claim 7 or 8 wherein said disease associated with the shortage of PGI₂ and/or the disease associated with the attenuation of the cPLA₂ function is heart infarction, angina pectoris, heart failure, pulmonary hypertension, hypertension, cerebrovascular disorder, arteriosclerosis obliterans, arteriosclerosis, hyperlipemia, diabetes, bronchial disease, stomach ulcer, eclampsia of pregnancy, hemolytic-uremic syndrome or thrombocytopenic purpura.

10. A pharmaceutical composition for the prevention or the treatment of a disease associated with the excess of PGI₂ and/or a disease associated with cPLA₂ hyperfunction, which comprises a CF6 activator or CF6 as the active ingredient.

11. The pharmaceutical composition for the prevention or

the treatment as claimed in claim 10 wherein said CF6 activator is a CF6 secretion accelerating substance or a CF6 agonist.

12. A pharmaceutical composition for the prevention or the treatment as claimed in claim 10 or 11 wherein said disease associated with the shortage of PGI₂ and/or a disease associated with cPLA₂ hyperfunction is an inflammatory disease such as brain infarction, acute pancreatitis, asthma, ARDS or rheumatoid arthritis.

13. A method of diagnosing a disease associated with an increase or decrease in the CF6 level in the blood, characterized in that the CF6 level in a collected blood sample is measured.

14. A diagnostic aid comprising an anti-CF6 antibody which is used in a method of diagnosing a disease associated with an increase or decrease in the CF6 level in the blood, characterized in that the CF6 level in a collected blood sample is measured.

15. The diagnostic aid as claimed in claim 14 wherein said anti-CF6 antibody is prepared by using the whole human CF6 (SEQ ID NO:1) or rat CF6 (SEQ ID NO:2) or a part thereof as an antigen.

16. A method of producing CF6 or a polypeptide which is a part thereof by using gene recombination techniques, which comprises culturing a host having been transformed by a vector comprising a DNA encoding a chimeric protein wherein a polynucleotide sequence encoding said CF6 or a partial polypeptide thereof is bonded to a protective peptide via a

polynucleotide sequence encoding an enterokinase recognition site at the N-terminus, and treating the thus obtained chimeric protein with enterokinase to thereby give CF6 or the partial polypeptide thereof.

17. A diagnostic method of judging the susceptibility to a disease associated with an increase or decrease in the CF6 level in the blood, which involves the step of determining the presence/absence of a variation in a gene sequence in the CF6 gene domain in the genome of a patient.

18. The diagnostic method as claimed in claim 17 wherein said disease with an increase or decrease in the CF6 level in the blood is a disease associated with the shortage or excess of PGI_2 .

19. The diagnostic method as claimed in claim 17 wherein said disease with an increase or decrease in the CF6 level in the blood is a disease associated with the accentuation or attenuation of cPLA_2 function.

20. The diagnostic method as claimed in claim 13 wherein said disease with an increase or decrease in the CF6 level in the blood is acute heart infarction.

Fig. 1

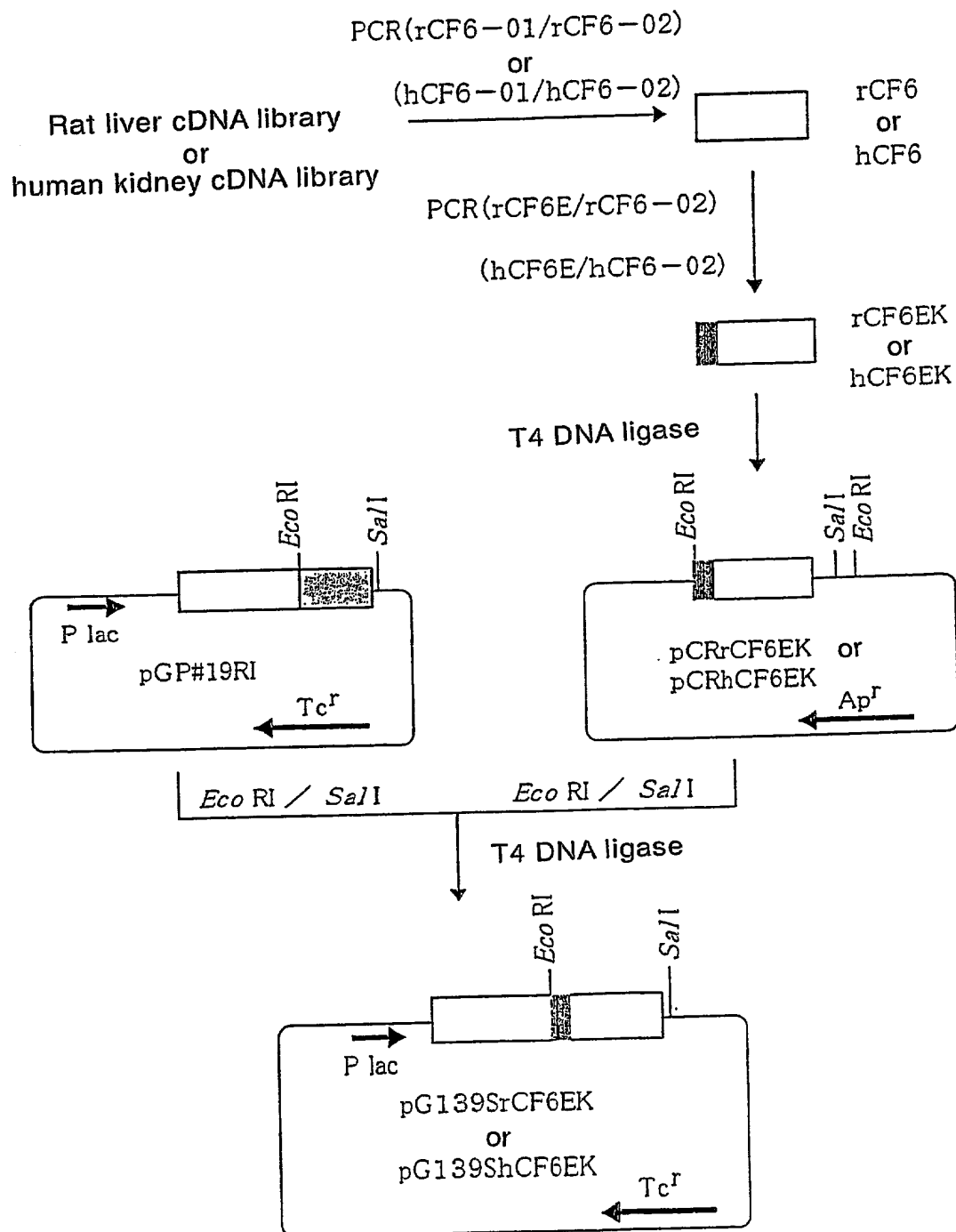


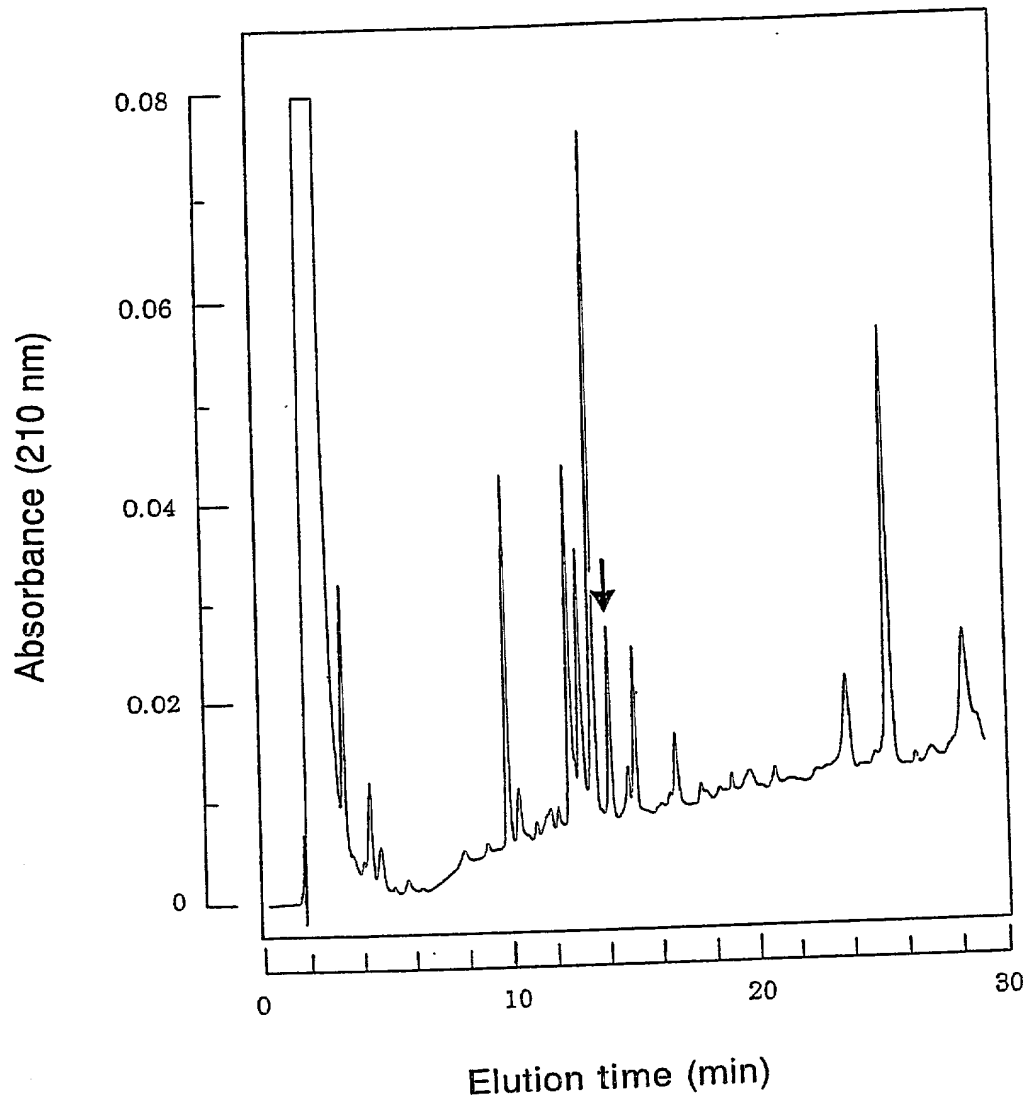
Fig. 2

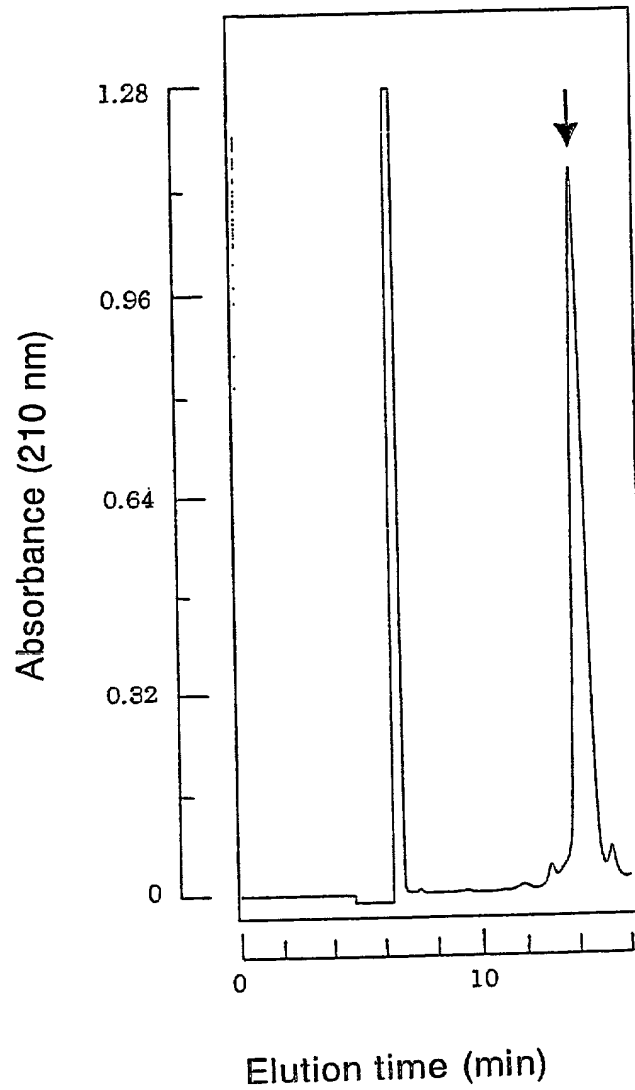
Fig. 3

Fig. 4

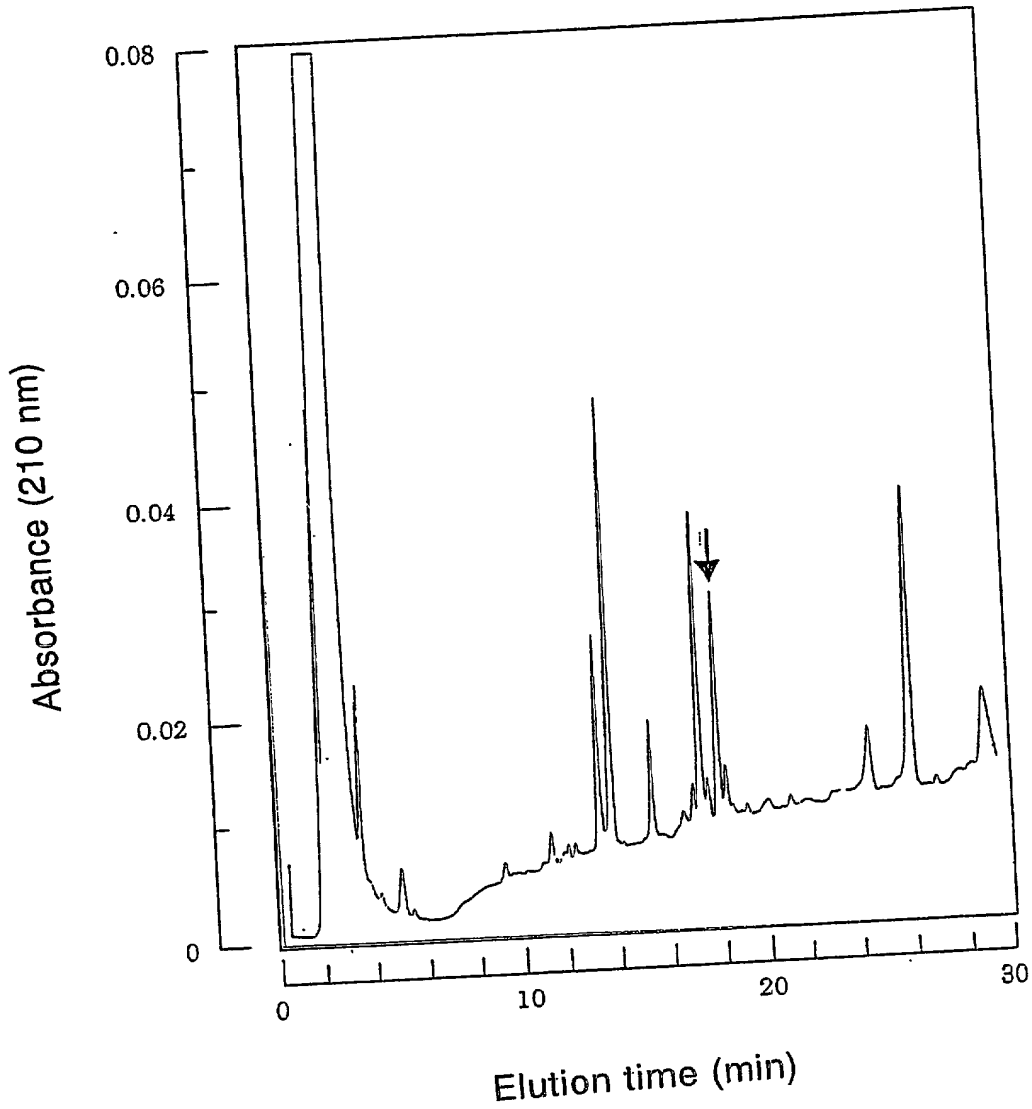


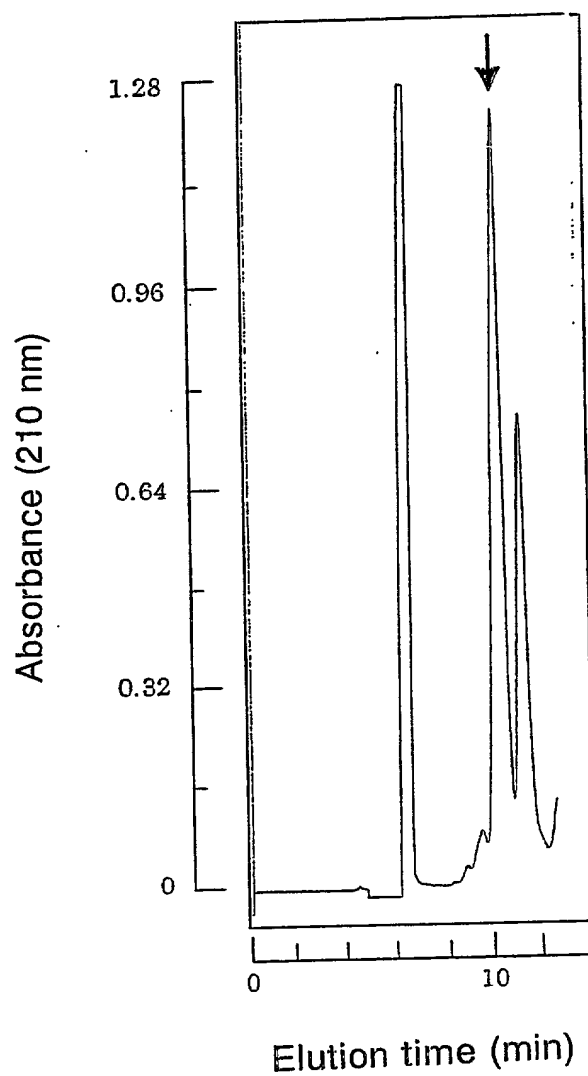
Fig. 5

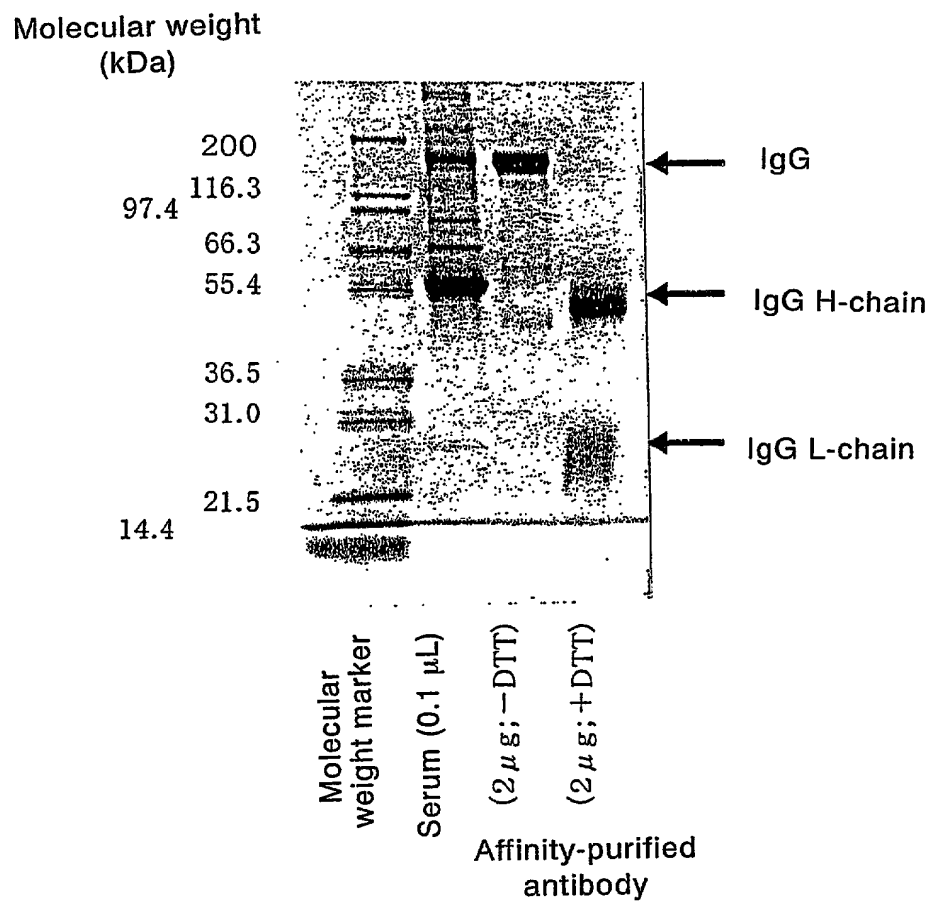
Fig. 6

Fig. 7

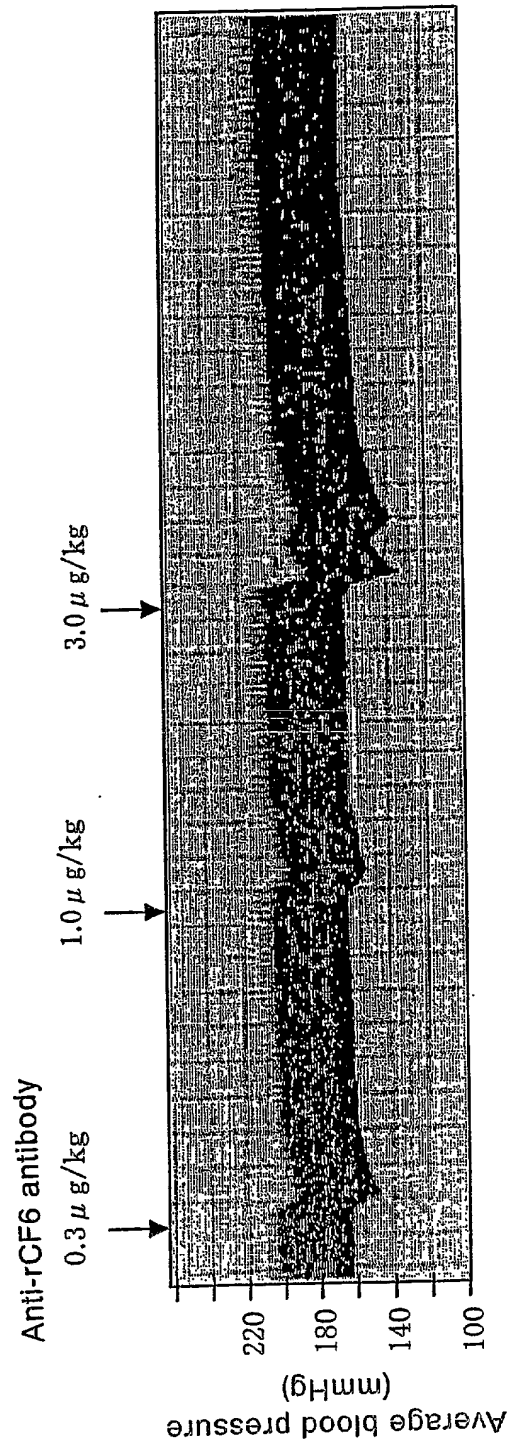


Fig. 8

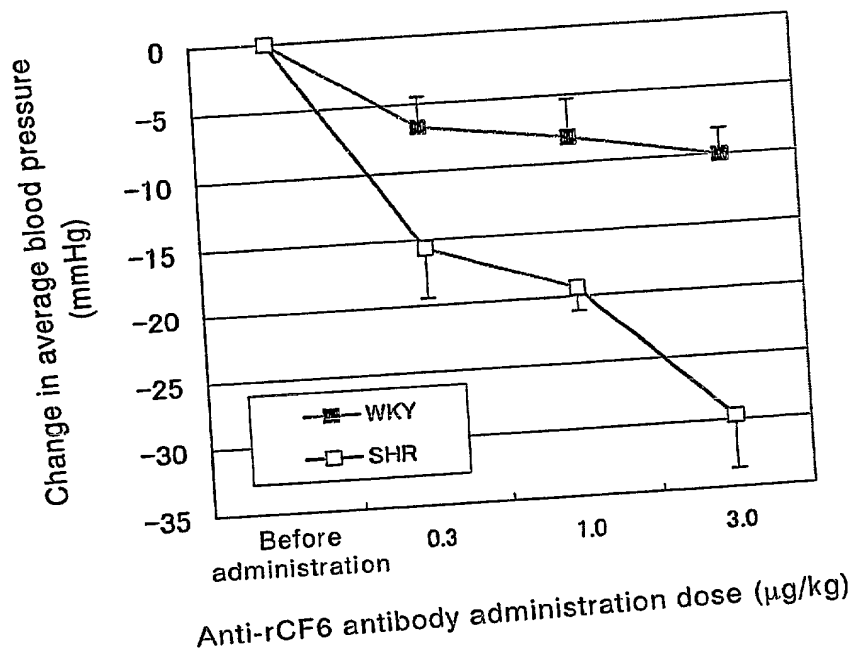
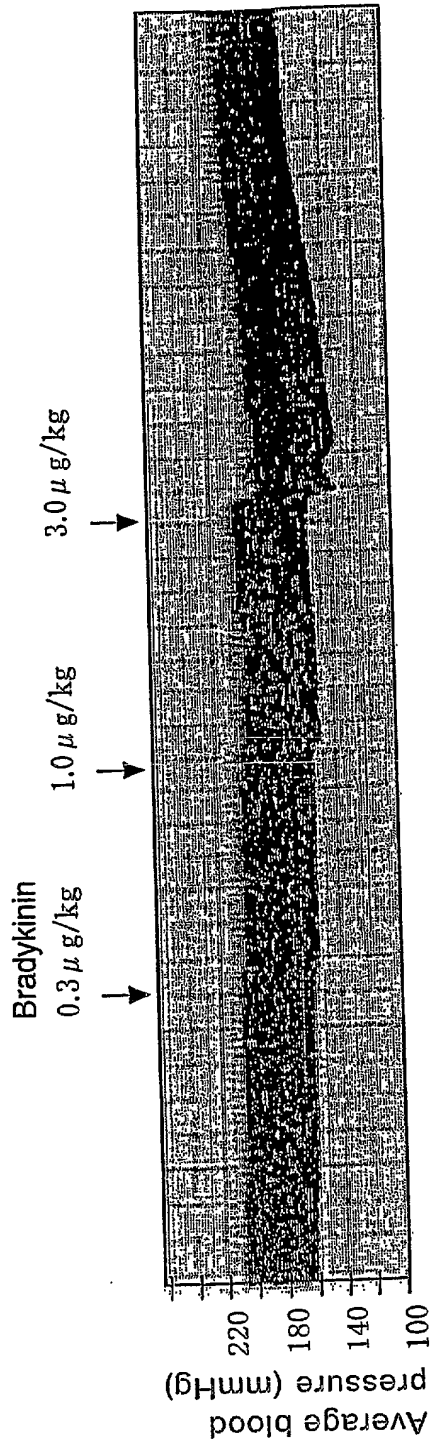


Fig. 9

1) Change in arterial blood pressure caused by the administration (iv) of bradykinin



2) Change in arterial blood pressure caused by the administration (iv) of bradykinin after the administration of anti-rCF6 antibody (3.0 μ g/kg, iv)

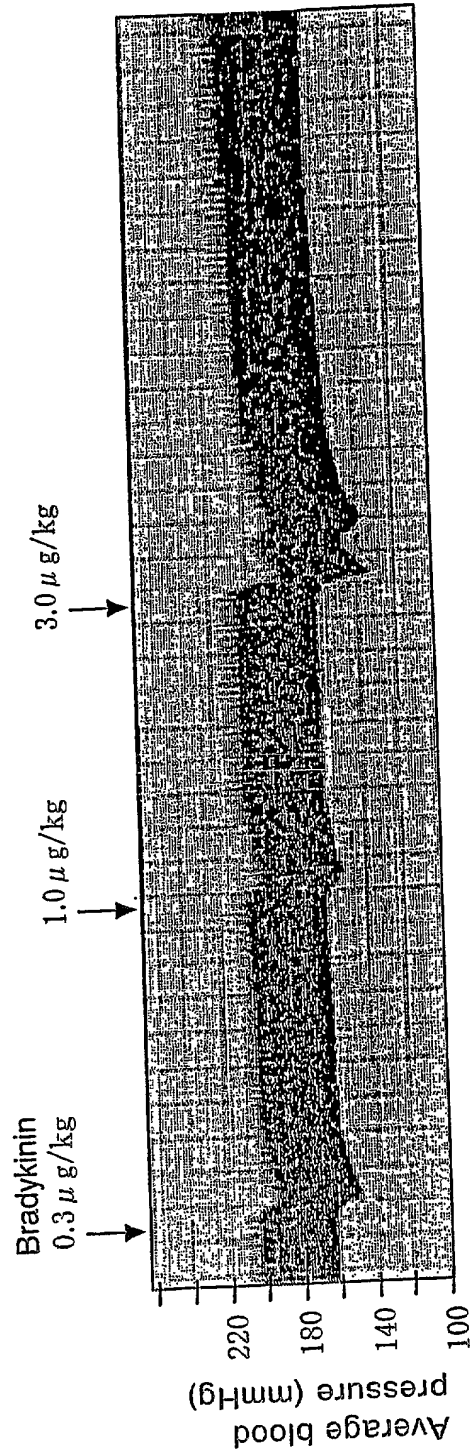


Fig. 10

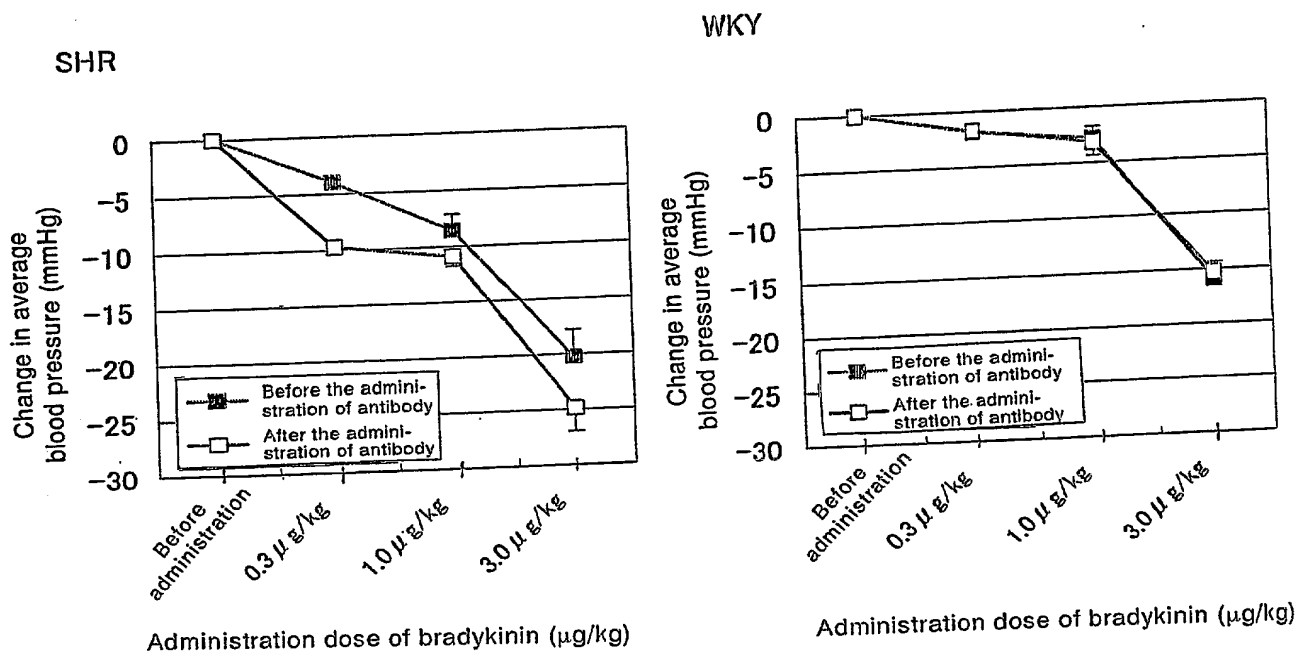


Fig. 11

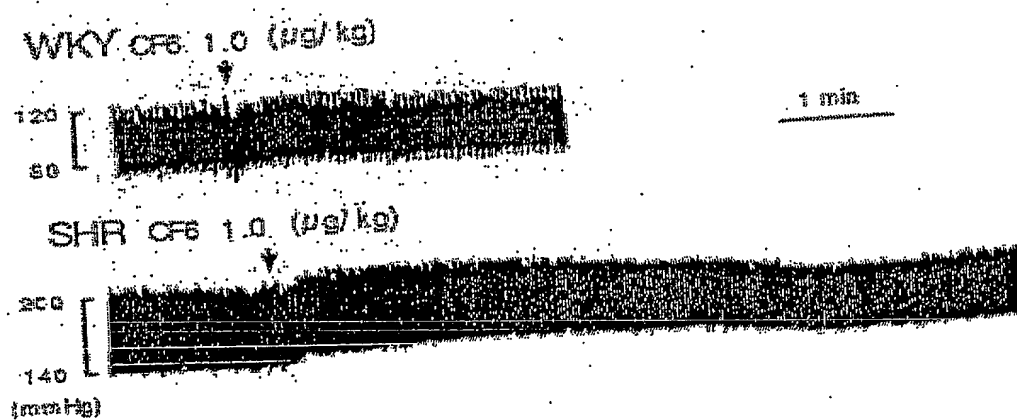


Fig. 12

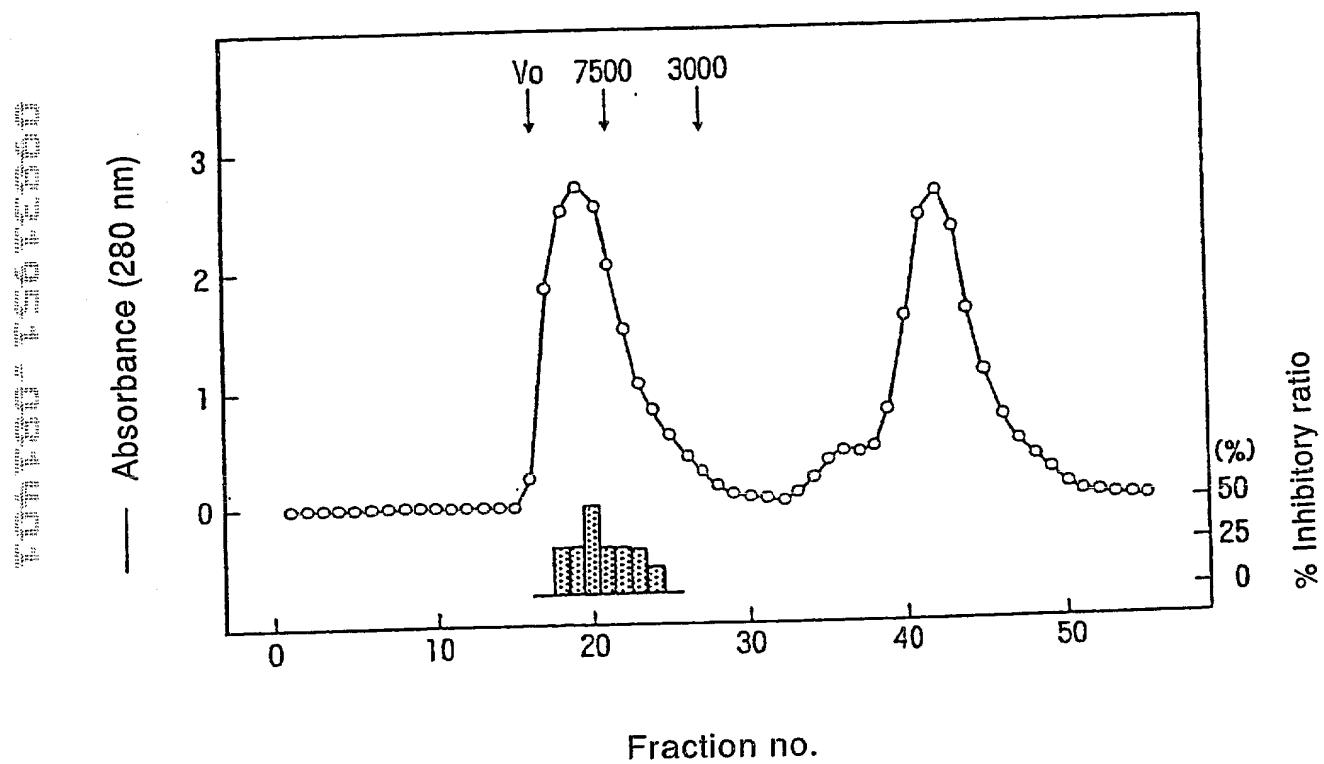
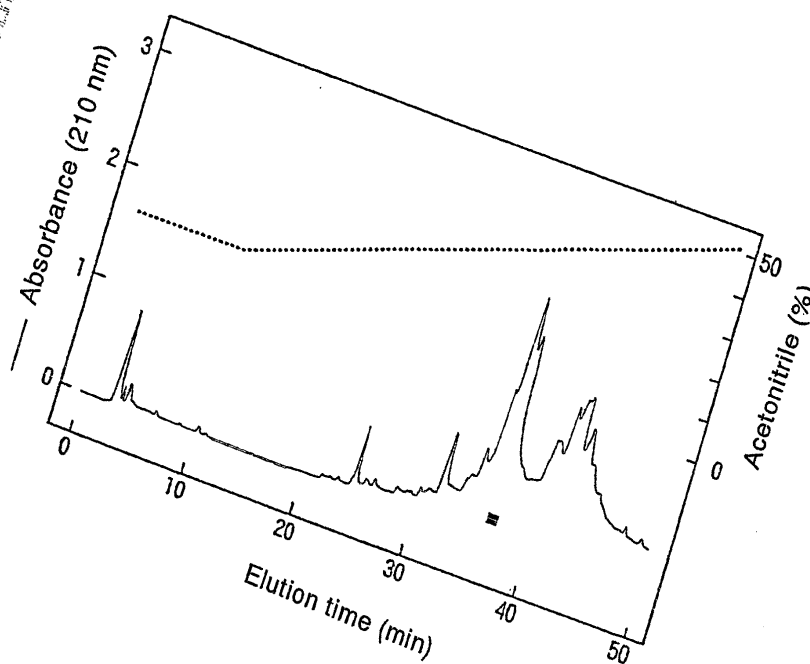


Fig. 13



09/831951

Fig. 14

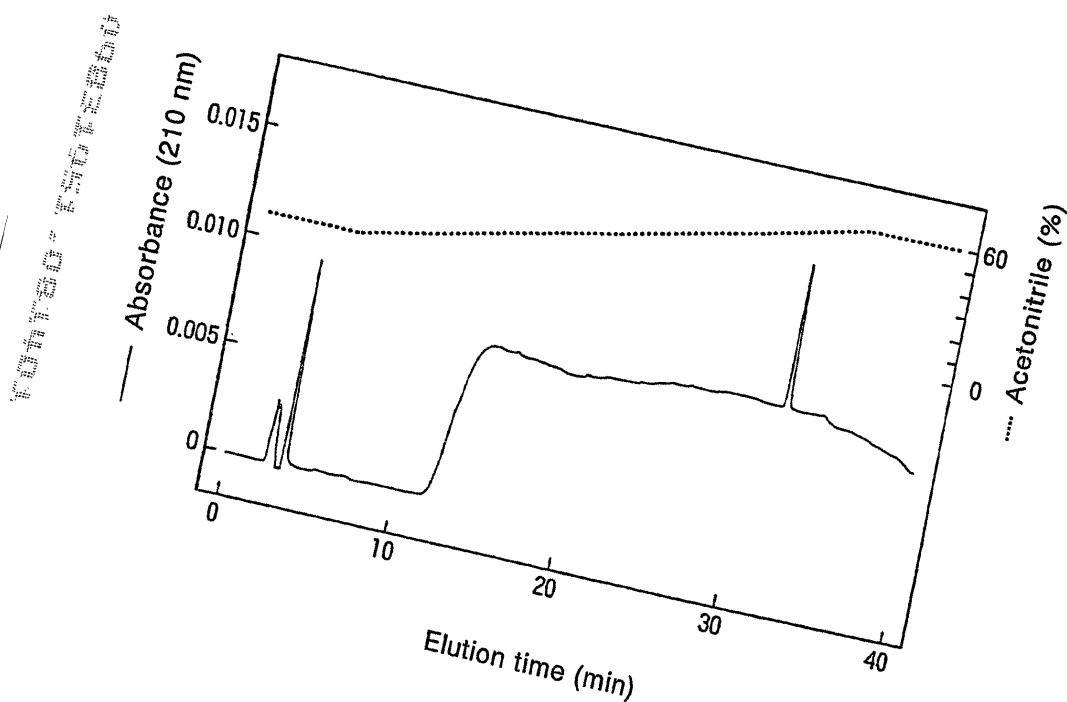


Fig. 15

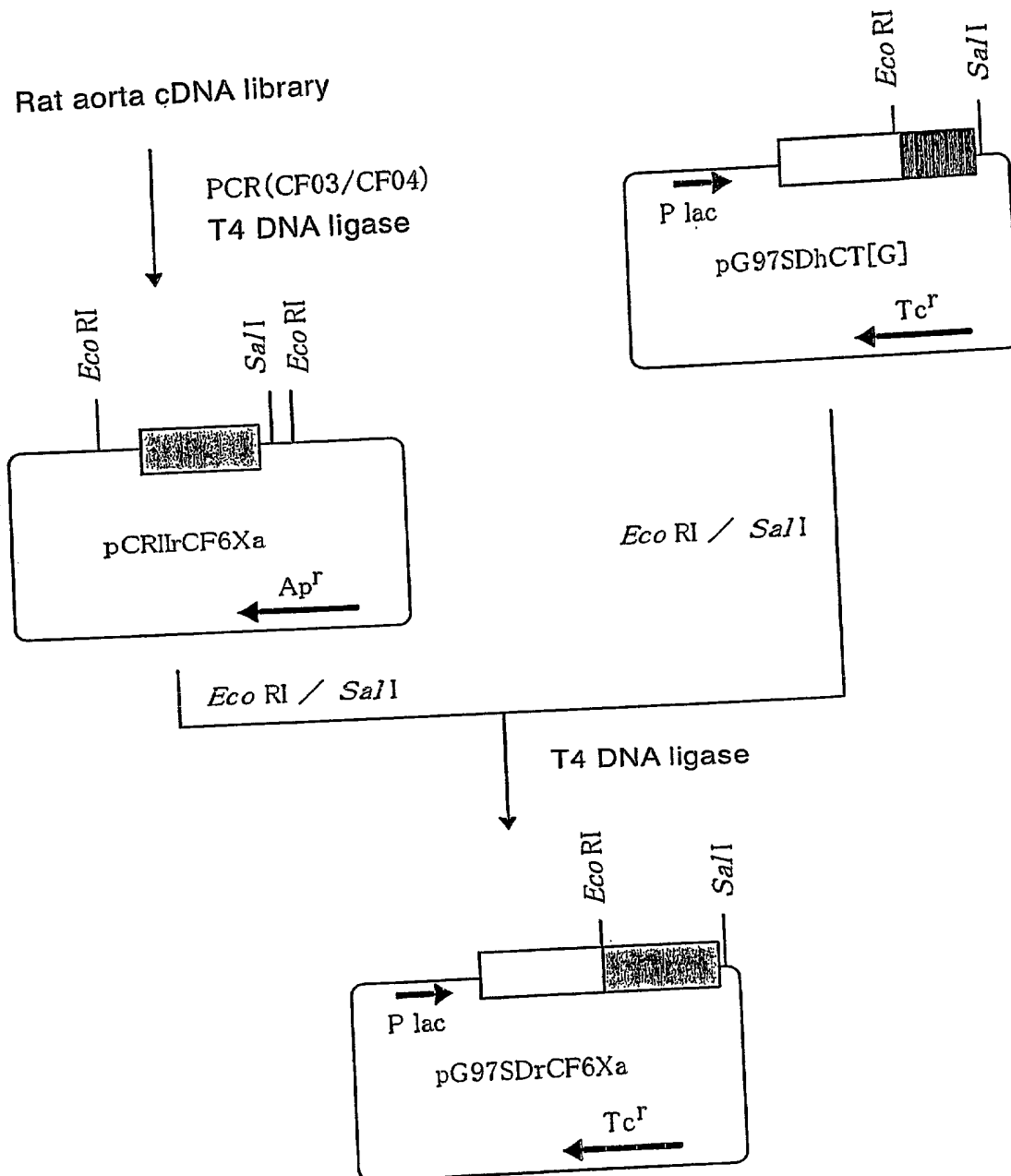


Fig. 16

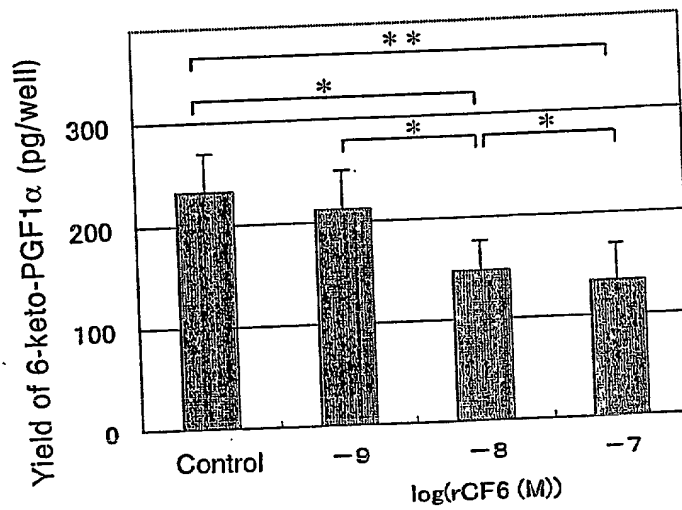


Fig. 17

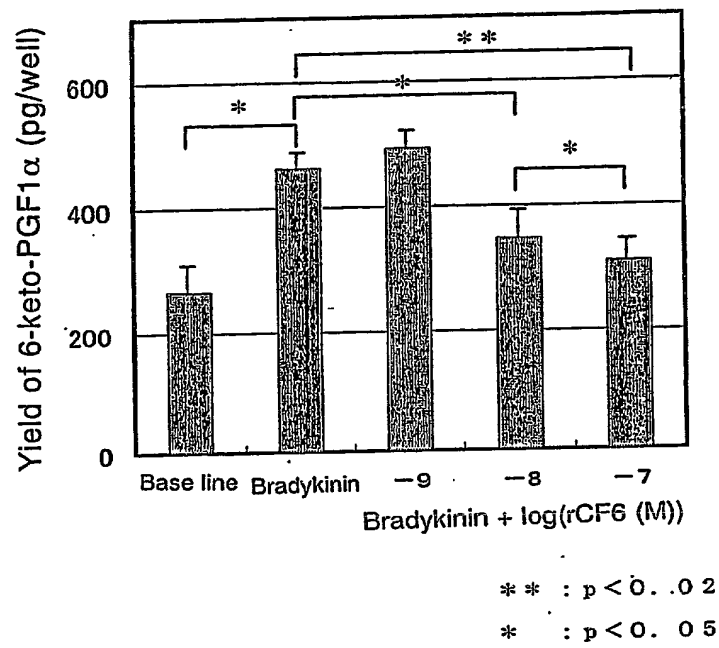


Fig. 18

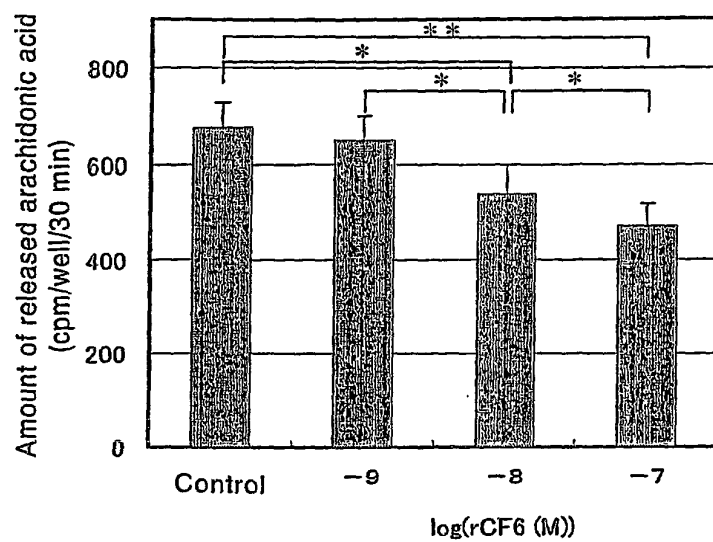
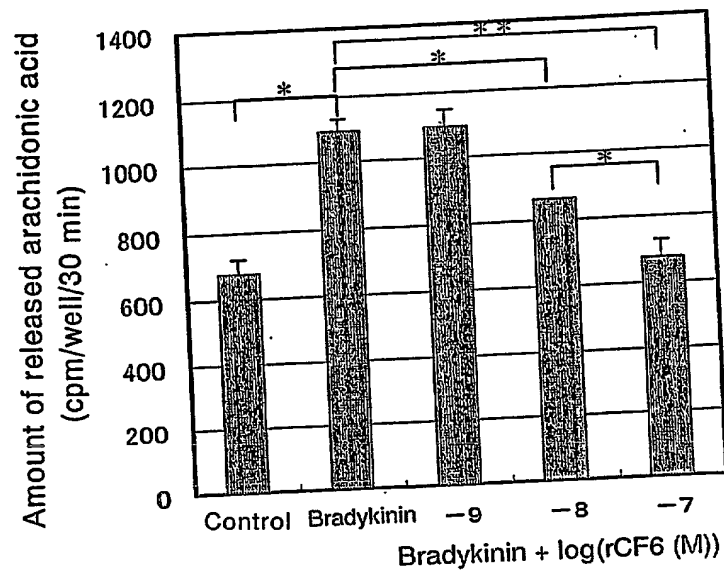


Fig. 19



** : $p < 0.01$

* : $p < 0.05$

Fig. 20

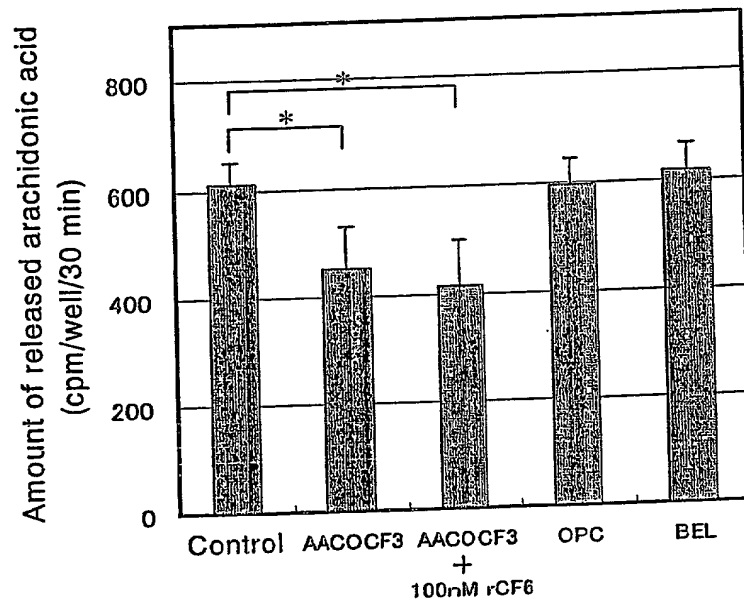


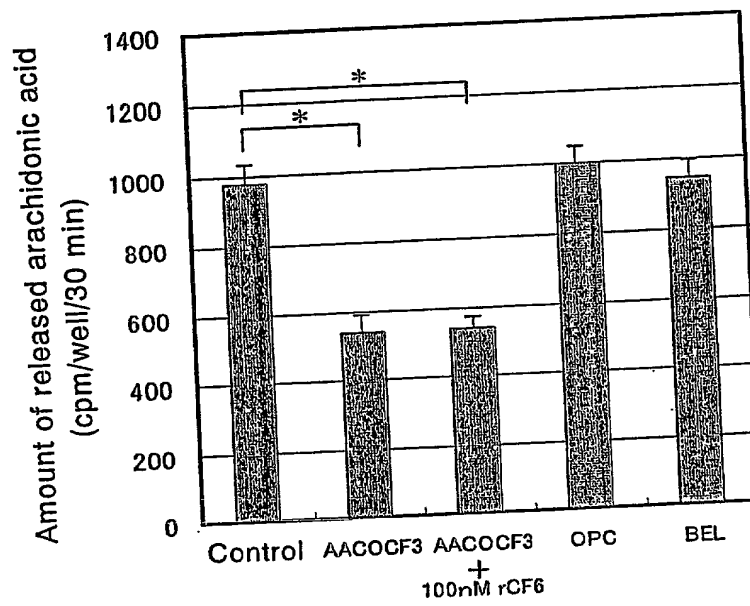
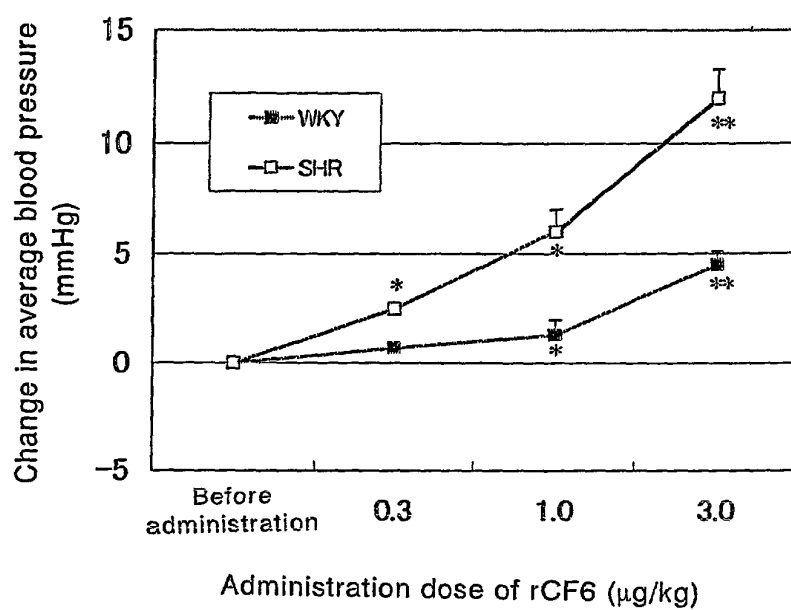
Fig. 21

Fig. 22

*p<0.05 vs. the level before administration

**p<0.01 vs. the level before administration

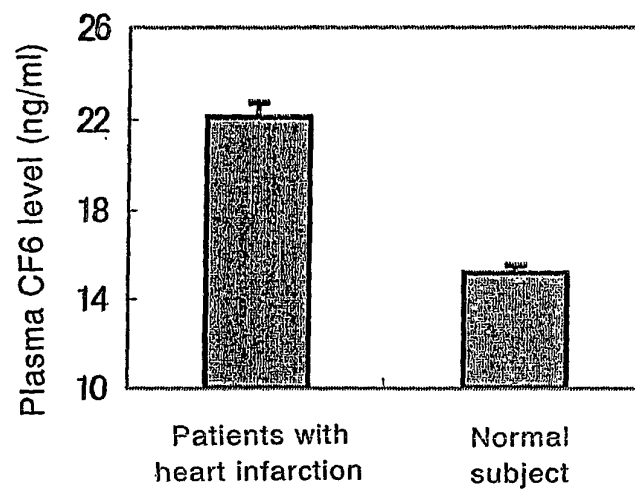
Fig. 23

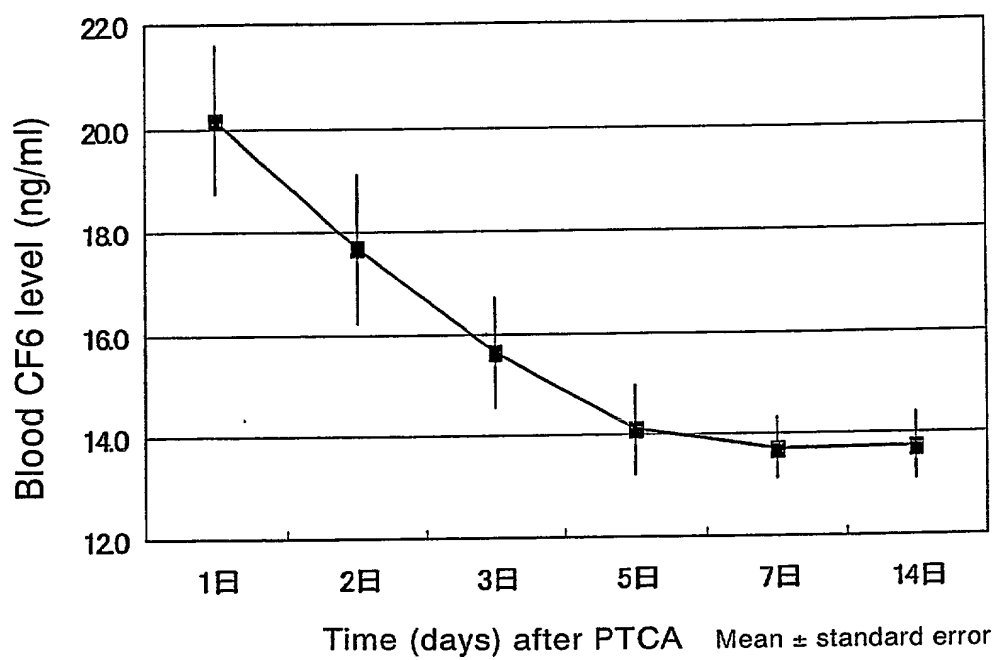
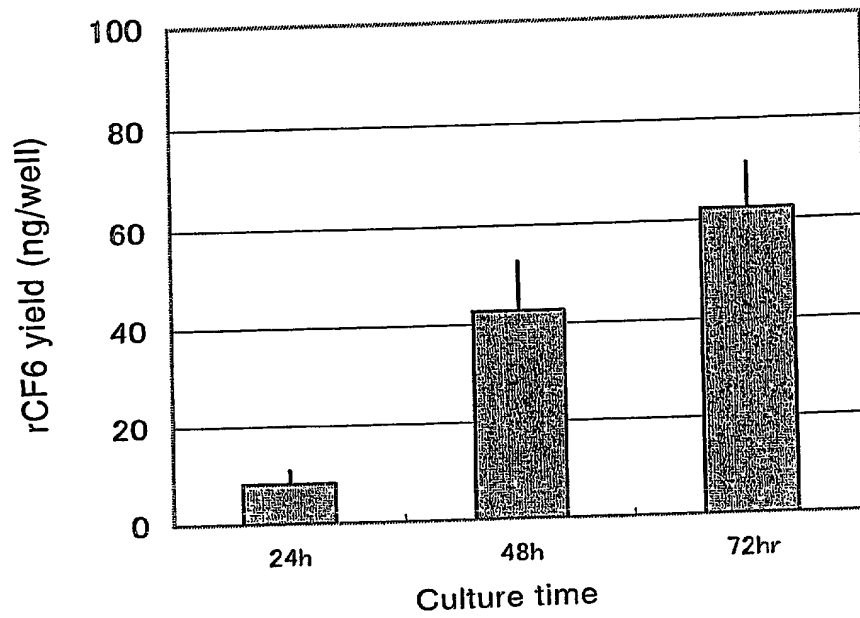
Fig. 24

Fig. 25

PCT/PCT Rec'd 14 AUG 2

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION IN THE
UNITED STATES PATENT AND TRADEMARK OFFICE

[] Declaration Submitted with Initial Filing or [X] Declaration Submitted after Initial Filing (surcharge 37 CFR 1.16 (e) required)

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the **INVENTION ENTITLED**
INHIBITOR AND ACTIVATOR FOR COUPLING FACTOR-6 AND USE THEREOF, the specification of which is:

[] attached hereto

OR

[X] was filed on (MM/DD/YYYY) 8/3/00 As United States Application Number (Attorney Docket No. _____) or
PCT International Application No. PCT/JP00/05210 and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 including for continuation-in-part application, material information which becomes available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a) -(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international Application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventors certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)			Priority Not Claimed	Certified Copy Attached?	
Number	Country	Foreign Filing Date (MM/DD/YYYY)		Yes	No
264687/1999	Japan	9/17/99	[]	[]	[X]

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional Application(s) listed below.

PRIOR U.S. PROVISIONAL(S)
Application No. (series code/serial no.) _____ Filing Date (MM/DD/YYYY) _____

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the registered practitioners represented by **Customer No.: 20736** to prosecute this application and transact all business in the U.S. Patent and Trademark Office in connection therewith. Direct all correspondence to **Manelli Denison & Selter PLLC at Customer No.: 20736**.

1. INVENTOR'S SIGNATURE: Tomohiro Osanai Date July 16, 2001
Inventor's Name (typed) Tomohiro OSANAI Japan
First Middle Initial Family Name Country of Citizenship
Residence (City) Nakatsugaru-gun (State) Aomori, Japan
Post Office Address (Include Zip Code) 31-1, Aza Muramoto, Oaza Komagoshi, Iwaki-machi, Nakatsugaru-gun,
Aomori 036-1322 Japan

2. INVENTOR'S SIGNATURE: Koji Magota Date July 16, 2001
Inventor's Name (typed) Koji MAGOTA Japan
First Middle Initial Family Name Country of Citizenship
Residence (City) Takatsuki-shi (State) Osaka, Japan
Post Office Address (Include Zip Code) 2-14-38, Okutenjin-cho, Takatsuki-shi, Osaka 569-1118 Japan

3. INVENTOR'S SIGNATURE: _____ Date _____
Inventor's Name (typed) _____
First Middle Initial Family Name Country of Citizenship
Residence (City) _____ (State) _____
Post Office Address (Include Zip Code) _____

[] Additional Inventors are being named on the _____ supplemental additional inventor sheet(s) attached hereto.

MDS Jan 2001

SEQUENCE LISTING

<110> SUNTORY LIMITED
<120> Inhibitor and Activator of Coupling Factor-6 and Antigen thereto
<130> YCT-515
<140> PCT/JP00/5210
<141> 2000-08-03
<150> JPA 264687/99
<151> 1999-09-17
<160> 24

<210> 1
<211> 76
<212> PRT
<213> Human
<400> 1

Asn Lys Glu Leu Asp Pro Ile Gln Lys Leu

1 5 10

Phe Val Asp Lys Ile Arg Glu Tyr Lys Ser

15 20

Lys Arg Gln Thr Ser Gly Gly Pro Val Asp

25 30

Ala Ser Ser Glu Tyr Gln Gln Glu Leu Glu

35 40

Arg Glu Leu Phe Lys Leu Lys Gln Met Phe

45 50

Gly Asn Ala Asp Met Asn Thr Phe Pro Thr

55 60
Phe Lys Phe Glu Asp Pro Lys Phe Glu Val

65 70
Leu Glu Lys Pro Gln Ala

75

<210> 2

<211> 76

<212> PRT

,<213> Rat

<400> 2

Asn Lys Glu Leu Asp Pro Val Gln Lys Leu

1 5 10

Phe Leu Asp Lys Ile Arg Glu Tyr Lys Ala

15 20

Lys Arg Leu Ala Ser Gly Gly Pro Val Asp

25 30

Thr Gly Pro Glu Tyr Gln Gln Glu Val Asp

35 40

Arg Glu Leu Phe Lys Leu Lys Gln Met Tyr

45 50

Gly Lys Gly Glu Met Asp Lys Phe Pro Thr

55 60

Phe Asn Phe Glu Asp Pro Lys Phe Glu Val

65 70

Leu Asp Lys Pro Gln Ser

75

85 90 95
 Ala Pro Ile Tyr Thr Asn Val Thr Tyr Pro Ile Thr Val Asn Pro Pro
 100 105 110
 Phe Val Pro Thr Glu Asn Pro Thr Gly Ser Tyr Ser Leu Thr Phe Asn
 115 120 125
 Val Asp Glu Ser Trp Leu Gln Glu Gly Gln Thr
 130 135

<210> 5

<211> 97

<212> PRT

<213> E. coli

<400> 5

Thr Met Ile Thr Asp Ser Leu Ala Val Val Leu Gln Arg Arg Asp Trp
 1 5 10 15
 Glu Asn Pro Gly Val Thr Gln Leu Asn Arg Leu Ala Ala His Pro Pro
 20 25 30
 Phe Ala Ser Trp Arg Asn Ser Asp Asp Ala Arg Thr Asp Arg Pro Ser
 35 40 45
 Gln Gln Leu Arg Ser Leu Asn Gly Glu Trp Arg Phe Ala Trp Phe Pro
 50 55 60
 Ala Pro Glu Ala Val Pro Asp Ser Leu Leu Asp Ser Asp Leu Pro Glu
 65 70 75 80
 Ala Asp Thr Val Val Val Pro Ser Asn Trp Gln Met His Gly Tyr Asp
 85 90 95
 Ala

<210> 6
<211> 23
<212> DNA
<213> Artificial Sequence
<220>
<221>
<222>
<223> Primer used in PCR method
<400> 6

atgactgttc agaggatctt cag

<210> 7
<211> 27
<212> DNA
<213> Artificial Sequence
<220>
<221>
<222>
<223> Primer used in PCR method
<400> 7

gtcgactcag gactgggggtt tgtcgag

<210> 8
<211> 23
<212> DNA
<213> Artificial Sequence
<220>
<221>

<222>

<223> Primer used in PCR method

<400> 8

atgattcttc agaggtctt cag

<210> 9

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<221>

<222>

<223> Primer used in PCR method

<400> 9

gtcgactcag gcctgggggtt ttctgatg

<210> 10

<211> 45

<212> DNA

<213> Artificial Sequence

<220>

<221>

<222>

<223> Gene coding for enterokinase recognition site and Eco RI recognition site

<400> 10

gaattcgacg atgacgataa gaataaggaa cttgatcctg tacag

<210> 11

<211> 46
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221>
 <222>
 <223> Gene coding for enterokinase recognition site and Eco RI recognition site
 <400> 11

gaattcgacg atgacgataa gaataaggaa cttgaccta tacaga

<210> 12
 <211> 20
 <212> PRT
 <213> rat
 <400> 12

Cys Phe Pro Thr Phe Asn Phe Glu Asp Pro Lys Phe Glu Val Leu

1 5 10 15

Asp Lys Pro Gln Ser

20

<210> 13
 <211> 20
 <212> PRT
 <213> rat
 <400> 13

Tyr Phe Pro Thr Phe Asn Phe Glu Asp Pro Lys Phe Glu Val Leu

1 5 10 15

Asp Lys Pro Gln Ser

20

<210> 14

<211> 19

<212> PRT

<213> human

<400> 14

Cys Leu Phe Val Asp Lys Ile Arg Glu Tyr Lys Ser Lys Arg Gln

1 5 10 15

Thr Ser Gly Gly

<210> 15

<211> 18

<212> PRT

<213> human

<400> 15

Leu Phe Val Asp Lys Ile Arg Glu Tyr Lys Ser Lys Arg Gln Thr

1 5 10 15

Ser Gly Gly

<210> 16

<211> 39

<212> PRT

<213> rat

<400> 16

Asn Lys Glu Leu Asp Pro Val Gln Lys Leu Phe Leu Asp Lys Ile

1 5 10 15

Arg Glu Tyr Lys Ala Lys Arg Leu Ala Ser Gly Gly Pro Val Asp

20 25 30

Thr Gly Pro Glu Tyr Gln Gln Glu Val

35

<210> 17

<211> 16

<212> PRT

<213> rat

<400> 17

Asp Arg Glu Leu Phe Lys Leu Lys Gln Met Tyr Gly Lys Gly Glu

1 5 10 15

Met

<210> 18

<211> 9

<212> PRT

<213> rat

<400> 18

Asp Lys Phe Pro Thr Phe Asn Phe Glu

1 5

<210> 19

<211> 7

<212> PRT

<213> rat

<400> 19

Asp Pro Lys Phe Glu Val Leu

1 5

<210> 20

<211> 5

<212> PRT

<213> rat

<400> 20

Asp Lys Pro Gln Ser

1 5

<210> 21

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<221>

<222>

<223> Factor Xa recognition site

<400> 21

Ile Glu Gly Lys

<210> 22

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<221>

<222>

<223> Primer for PCR method

<400> 22

gatcgagggacgtaataaggaacttgatcct

<210> 23

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<221>

<222>

<223> Primer for PCR method

<400> 23

gtcgacttaggactgggggttgcga

<210> 24

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<221>

<222>

<223> Factor Xa recognition site containig peptide

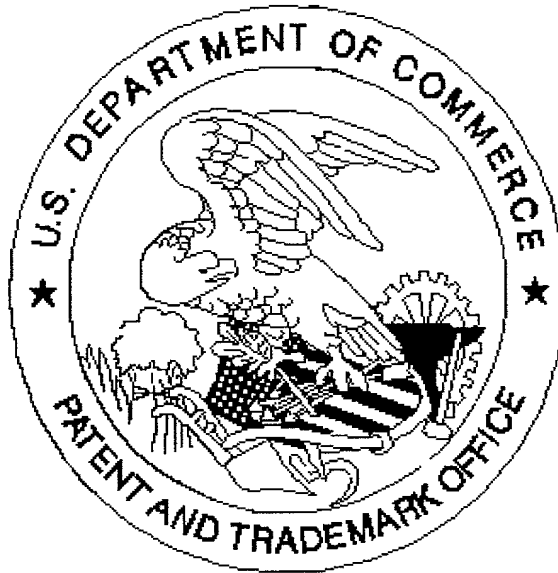
<400> 24

Glu Phe Gly Leu Ile Glu Gly Lys

1

5

United States Patent & Trademark Office
Office of Initial Patent Examination -- Scanning Division



Application deficiencies found during scanning:

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☐ *Scanned copy is best available. some drawing are dark*